Improvements in the Tolerance of Photosystem II to Photo-Oxidative Stress in Cyanobacteria

A Thesis

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by

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Abstract

Effects of overexpression of superoxide dismutase and catalase on the tolerance of photosystem II (PSII) to photo-oxidative stress were studies in the cyanobacterium *Synechococcus elongatus* PCC 7942.

Chapter 1 describes the photosynthetic process, photo-oxidative stress, and the antioxidative systems in photosynthetic organisms that include plants and cyanobacteria. Effects of photo-oxidative stress on cyanobacteria, as well as other photosynthetic organisms, and on their photosynthetic machinery under environmental stresses are reviewed. Reactive oxygen species (ROS) that are generated as by-products during photosynthetic reactions are reviewed. Each type of ROS is described in details in term of their properties and detoxifying processes. In addition, the defense mechanisms in cyanobacteria against ROS are described. The aim of the present study is to enhance the tolerance of photosystem II to photo-oxidative stress by improving the antioxidative systems with a special focus on ROS-scavenging enzymes.

Chapter 2 describes the effects of overexpression of superoxide dismutase (SOD) and catalase on photosystem II (PSII) under photo-oxidative stress in the cyanobacterium *Synechococcus elongatus* PCC 7942. In the present study, an iron superoxide dismutase (Fe-SOD) from *Synechocystis* sp. PCC6803; a highly active catalase (VktA) from *Vibrio rumoiensis*; and both enzymes together were overexpressed. Then I examined the sensitivity of PSII to photoinhibition in the three strains. In cells that overexpressed either Fe-SOD or VktA, PSII was more tolerant to strong light than it was in wild-type cells. Moreover, in cells that overexpressed both Fe-SOD and VktA, PSII was even more tolerant to strong light. However, the rate of photodamage to PSII, as monitored in the presence of chloramphenicol, was similar in all three transformant stains and in wild-type cells, suggesting that the

overexpression of these ROS-scavenging enzymes might not protect PSII from photodamage but might protect the repair of PSII. Under strong light, intracellular levels of ROS fell significantly, and the synthesis *de novo* of proteins that are required for the repair of PSII, such as the D1 protein, was enhanced. These observations suggest that overexpressed Fe-SOD and VktA might act synergistically to alleviate the photoinhibition of PSII by reducing intracellular levels of ROS, with resultant protection of the repair of PSII from oxidative inhibition.

Chapter 3 describes the conclusions from the present study and the perspectives of future study. Future study should be directed towards fuller understanding of the mechanisms by which the overexpression of Fe-SOD and VktA enhances the synthesis of the D1 protein. Effects of the overexpression of Fe-SOD and VktA on other antioxidative systems also remain to be elucidated. The established strategy with Fe-SOD and VktA to protect photosynthesis from photo-oxidative stress should also be applied to improvements in the tolerance of photosynthetic organisms to various types of environmental stress.

Abbreviations

Carboxy-H_2DCFDA	5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate
CBB	Coomassie Brilliant Blue
Chl	Chlorophyll
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
MV	Methyl viologen
NBT	Nitro blue tetrazolium
OEC	Oxygen-evolving complex
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase

Chapter 1

General Introduction

1.1 Photosynthesis

Photosynthesis is a biochemical process that produces sugars from carbon dioxide and requires light as the source of energy. Photosynthesis consists of multiple steps of consecutive redox reactions that occur upon absorption of light energy and transfer the energy through electron-transport reactions in the photosynthetic machinery. The photosynthetic activity is important for not only photosynthetic organisms, such as higher plants, algae, diatoms, and cyanobacteria but also most of organisms on the earth to provide carbon sources. Photosynthetic organisms are the primary colonizers of the ecosystem and the oxygen generators. Moreover, photosynthesis is responsible for the productivity and biomass of photosynthetic organisms: more than 90% biomass of rice mainly comes from photosynthetic products and the grain yield of rice can be increased by enhancing the photosynthetic efficiency. To improve the photosynthetic systems, it is necessary to understand the mechanisms of photosynthetic reactions and, in particular, of the responses of photosynthesis to environmental changes.

1.1.1 Photosynthesis in cyanobacteria

Cyanobacteria (formerly known as blue-green algae) are in the kingdom of monera and the division of cyanophyta, which comprises a large group within the prokaryotic kingdom. They are among the oldest oxygenic photosynthetic organisms of life since 3.5 billion years (Khan et al. 2005). Cyanobacteria have long been known for their ecological and agricultural impacts, as the primary colonizers of the ecosystem that are able to fix atmospheric carbon dioxide.

The photosynthetic apparatus of cyanobacteria is similar to that of higher plants, and the chloroplasts of plants are believed to originate from cyanobacteria. Cyanobacteria can

perform oxygenic photosynthesis and respiration simultaneously in the same comartment. Photosynthetic electron transport occurs in the thylakoid membranes, while respiratory electron transport occurs in both thylakoid and cytoplasmic membranes (Vermaas 2001). In some cyanobacteria, such as *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, their thylakoid membranes are arranged in concentric layers, following the shape of the cell. In other cyanobacteria, such as *Cyanothece* sp. PCC 51142, thylakoid membranes form a dense network that spread all over the cell. This thylakoid membrane network is formed from the branching and splitting of membranes and encloses a single luminal space. The structures of thylakoid membranes in these cyanobacteria considerably differ from those of thylakoid membranes in plants. Thylakoid membranes in plants are organized in interconnected stacked grana and unstacked stroma (Liberton et al. 2011).

Photosynthetic process in cyanobacteria is similar to that in plants, even though some of constituents of photosynthetic apparatus are different. The major complexes that drive photochemical reactions in cyanobacteria consist of phycobilisomes, photosystem II (PSII), cytochrome b_6/f (Cyt b_6/f), photosystem I (PSI) (Fig. 1.1) (Nagarajan and Pakrasi 2001). The protein composition of each complex is shown in Table 1.1.

Cyanobacteria have been used as model organisms in many studies for photosynthesis. *Synechococcus elongatus* PCC 7942 is a unicellular, rod-shaped cyanobacterium (Fig. 1.2), which is extensively used in various studies that include photosynthesis, antioxidative systems, and environmental stress acclimation. This species is easily transformable and grows fast and its genome (2.7 Mb) has been fully sequenced (Holtman et al. 2005).

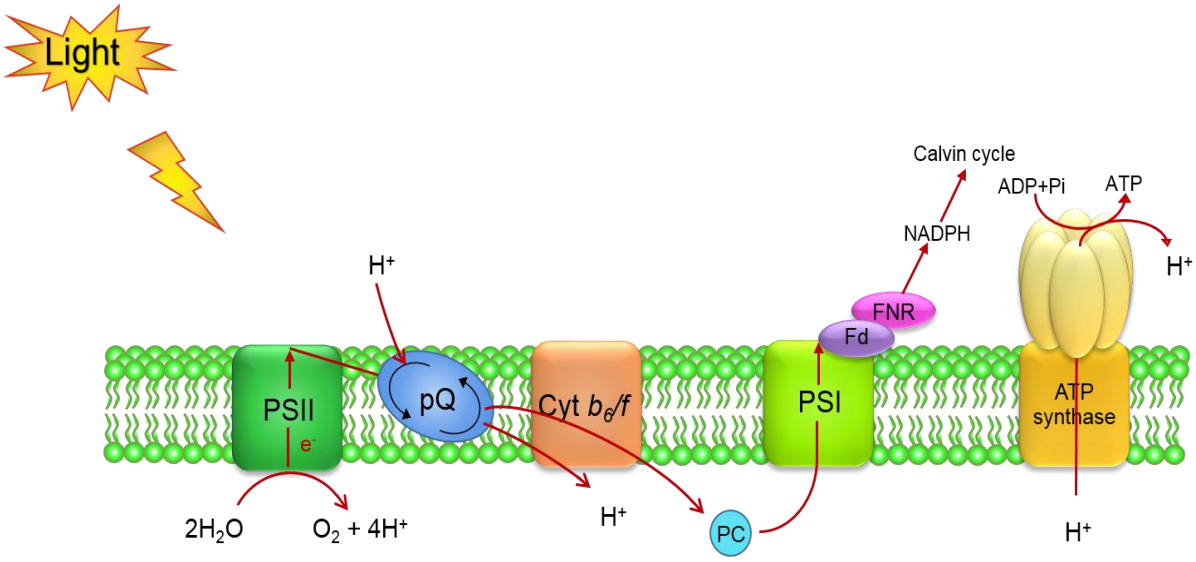

Figure 1.1 Simplified scheme representing the photosynthetic components.

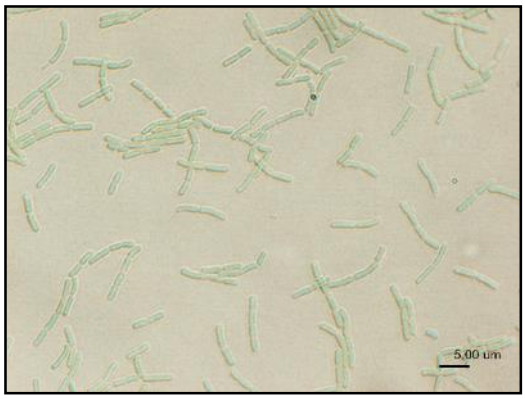

Figure 1.2 Morphology of *Synechococcus elongatus* PCC7942 observed with optical microscopy.

Table 1.1 Major complexes involved in the photosynthetic machinery in thylakoid membranes in cyanobacteria. Adapted from (Vermaas 2001).

Complex	Gene designation	Major proteins	Cofactors	Functions
Photosystem II	*psb*	D1, D2, CP43, CP47, PsbO	Mn, Ca, Cl, Fe, PQ, chlorophyll, cyt$_{b559}$	Light-induced water splitting and PQ reduction
Photosystem I	*psa*	PsaA, PsaB and other Psa proteins	Chlorophyll, vitamin K_1, FeS centres	Light-induced PC/cyt c_{553} oxidation and Fd reduction
Cytochrome *b6f*	*pet*	cyt b_6, cyt *f*, Rieske, subunit IV	2 cyt *b*,cyt *f* (cyt *c*), FeS	PQH_2 oxidation and PC/cyt c_{553} reduction

1.2 Photo-oxidative stress

Photosynthetic organisms are susceptible to various environmental stresses, including high salinity, drought, low-high temperature, nutrient deficiency, heavy metals, and strong light. Of these stresses, strong light is the major factors that causes photo-oxidative stress. In nature, photo-oxidative stress often occurs when strong light is combined with other types of stress, such as salt stress and high-temperature stress. These environmental stresses induce the generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•). In general, organisms have antioxidative systems to scavenge these ROS. However, when the production of ROS exceeds the capacity of their scavenging, cells extremely suffer from oxidative damage. Oxidative damage affects the structure and function of DNA, membrane and protein, leading to the suppression of growth and productivity of photosynthetic products (Fig. 1.3) (Freinbichler et al. 2011).

In addition, photo-oxidative stress causes the specific inactivation of PSII, and this phenomenon is referred to as photoinhibition of PSII (Powles 1984, Aro et al. 1993). Decrease in the activity of PSII results in the decrease in the overall photosynthetic activity. To cope with photoinhibition, photosynthetic organisms have evolved various antioxidative systems.

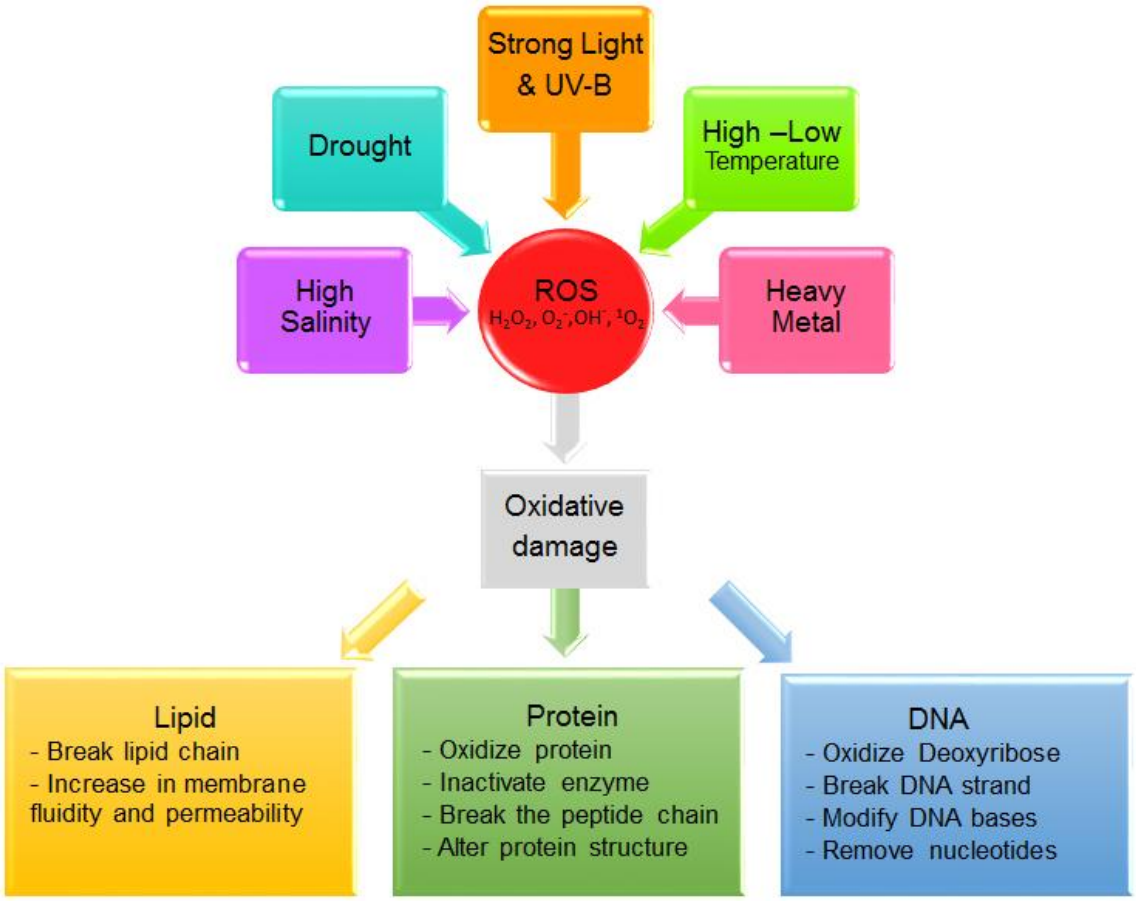

Figure 1.3 Various environmental stresses induce the generation of ROS in photosynthetic organisms, which leads to damage to biomolecules in cells.

1.2.1 Photoinhibition of PSII

Photosynthesis is sensitive to various types of environmental stress, and strong light is one of the most significant environmental stressors. Exposure of photosynthetic organisms to strong light often results in the specific inactivation of photosystem II (PSII), and this phenomenon is referred to as photoinhibition of PSII (Powles 1984, Aro et al. 1993). In living cells, PSII is damaged by light and, almost simultaneously, it is repaired by a rapid repair system (Aro et al. 1993, Aro et al. 2005). Photoinhibition of PSII occurs when the rate of light-induced damage (photodamage) to PSII exceeds the rate of repair of photodamage of PSII (Fig. 1.4) (Aro et al. 1993, Aro et al. 2005). The rate of photodamage to PSII is proportional to light intensity, whereas the rate of repair of PSII reaches a plateau under light at relatively weak intensities (Park et al. 1995, Tyystjärvi and Aro 1996, Anderson and Chow 2002, Allakhverdiev and Murata 2004). To gain better understanding of the nature of photoinhibition, it is necessary to study the processes of photodamage and repair separately, and methods for the separate examination of these processes have been established in cyanobacteria and plants (Gombos et al. 1994, Wada et al. 1994, Moon et al. 1995).

Examination of photodamage and repair separately can be achieved by using antibiotics, such as chloramphenicol and lincomycin, which inhibit the repair of PSII via the inhibition of protein synthesis (Nishiyama et al. 2001, Takahashi and Murata 2005, Hakkila et al. 2014). Moreover, the role of ROS in photoinhibition of PSII can be studied by this approach (Murata et al. 2012, Nishiyama and Murata 2014). Better understanding of the mechanism of photoinhibition will provide the methods to improve the photosynthetic processes to alter the growth and productivity of photosynthetic organisms.

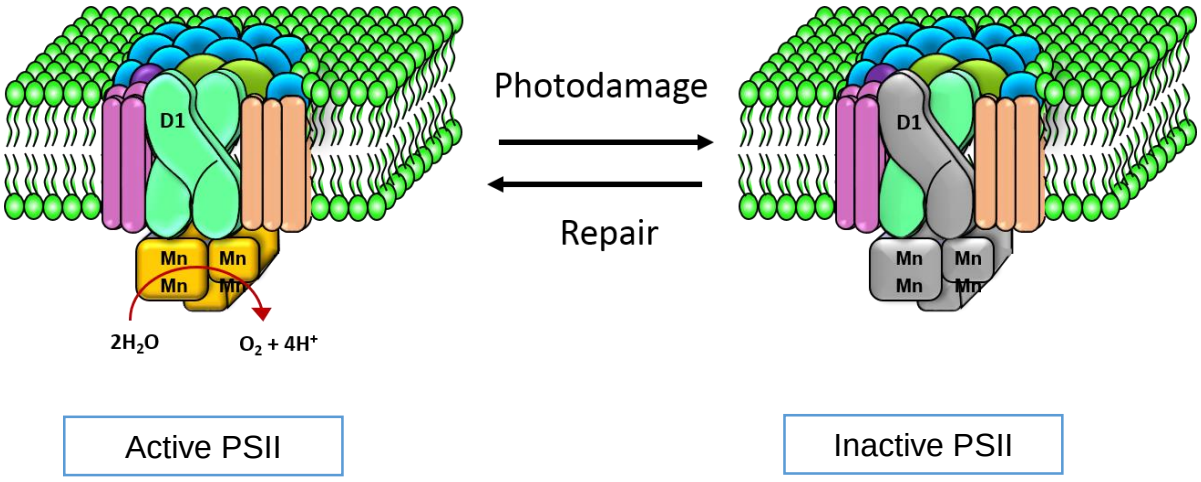

Figure 1.4 A model for photodamage to and the repair of PSII under strong light.

1.2.1.1 Photodamage to PSII

Light is the main source of energy for oxygenic photosynthesis, but it is also harmful to the photosynthetic machinery, in particular, to PSII. PSII is highly sensitive to strong light and can be inactivated. The rate of photodamage is proportional to the light intensity (Tyystjärvi and Aro 1996, Nishiyama et al. 2001, Allakhverdiev and Murata 2004) and depends on the wavelength of light (Jones and Kok 1966, Hakala et al. 2005, Takahashi et al. 2010).

During the photosynthetic reactions, ROS are inevitably produced as by-products and the production of ROS is highly accelerated under strong light. Earlier studies of photodamage to PSII hypothesized that ROS and, in particular, $^1O_2$ is the mediator of photodamage to PSII. $^1O_2$ is generated from energy transfer via triplet state of chlorophylls during charge recombination at the reaction center, as follows. Excess light energy affects the reaction center of PSII, resulting in the inhibition of electron transfer from Q_A^- (the first quinone acceptor) to Q_B (the second quinone acceptor). Then, an increase in charge recombination of the radical pair, $P680^+/Pheo^-$ in the reaction center of PSII promotes the formation of triplet state of chlorophylls (^{3}Chl). ^{3}Chl interacts with oxygen molecules, yielding $^1O_2$, which directly damages the D1 protein, a major subunit that constitutes the reaction center of PSII (Fig. 1.5). These hypotheses are represented by the "acceptor-side" and "charge-recombination" models (Vass et al. 1992, Hideg et al. 1994, Keren et al. 1997). However, most of earlier studies that supported these models were performed under conditions *in vitro*, and thus lack of examinations of repair.

Separate examination of photodamage and repair revealed that ROS act primarily by inhibiting the repair of PSII and not by damaging PSII directly (Nishiyama et al. 2001, Nishiyama et al. 2004). In addition, increased production of ROS in the culture of *Synechocystis* by addition of H_2O_2 and methyl viologen enhanced the extent of photoinhibition. The enhanced photoinhibition was attributed to the inhibition of the repair of photodamaged

PSII but not to the acceleration of photodamage to PSII (Nishiyama et al. 2001, Allakhverdiev and Murata 2004). Similarly, increased production of $^1O_2$ by addition of photosensitizers, such as rose bengal and ethyl eosin, decreased the rate of repair of photodamaged PSII, but did not affect the rate of photodamage to PSII (Nishiyama et al. 2004). These studies indicate that photodamage of PSII might not be associated with ROS including $^1O_2$, and that ROS might not be responsible for the primary damage to PSII.

Recent studies proposed a new model for photodamage that is referred to as the "two-step" model (Fig. 1.6) (Ohnishi et al. 2005). In this model, photodamage to PSII occurs in two sequential steps. Firstly, the oxygen-evolving complex and, most probably the manganese cluster, is damaged via absorption of UV and blue light (Ohnishi et al. 2005). This damage step is consistent with the action spectrum of photodamage to PSII that resembles the absorption spectrum of manganese compounds (Hakala et al. 2006). Then, the damage to the oxygen-evolving complex triggers damage to the reaction center via absorption of visible light that has been absorbed by chlorophylls (Ohnishi et al. 2005). In addition, the manganese cluster can also absorb visible light even though the extent of absorption is much less. However, it is sufficient to damage the oxygen-evolving complex at the first step of photodamage to PSII (Zavafer et al. 2015).

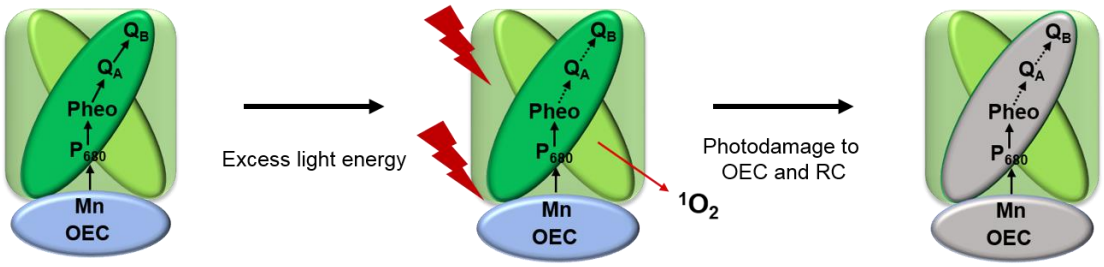

Figure 1.5 The model for photodamage to PSII represented by "acceptor-side" and "charge-recombination" mechanisms. $^1O_2$ that is produced via the transfer of excitation energy directly damages the reaction center (RC).

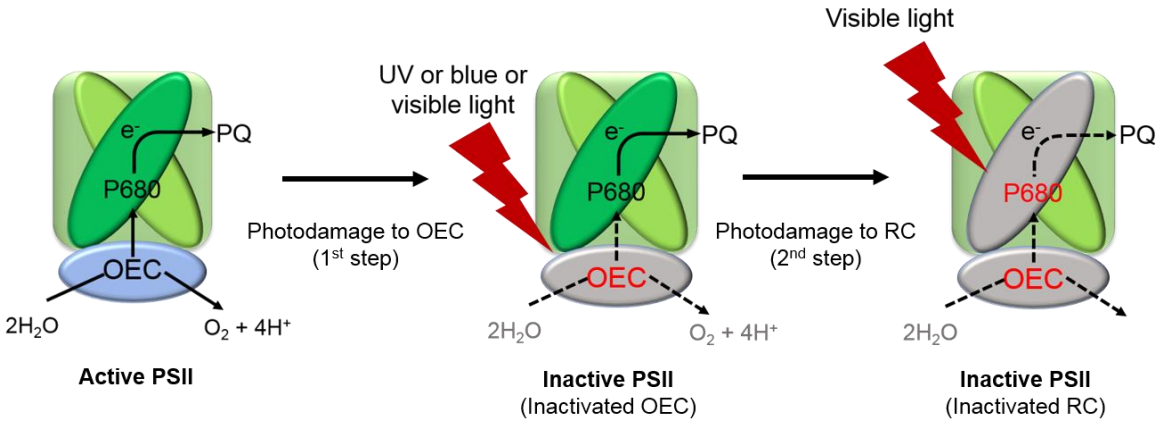

Figure 1.6 The "two-step" model for photodamage to PSII. Photodamage to PSII occurs in two sequential steps. Firstly, the oxygen-evolving complex (OEC) is damaged by light and, in particular, UV and blue light. The damage to OEC triggers damage to the reaction center (RC) upon absorption of visible light. PQ, plastoquinone.

1.2.1.2 The repair of PSII

In living organisms, photodamaged PSII is repaired by a rapid and efficient repair system. The damaged D1 protein is immediately degraded by proteases (Lindahl et al. 1996, Lindahl et al. 2000, Haussuhl et al. 2001) and replaced by a newly synthesized D1 protein (Aro et al. 1993). The repair of PSII occurs in several steps, as follows: proteolytic degradation of the D1 protein; synthesis *de novo* of the precursor to D1 (known as pre-D1); insertion of pre-D1 into the thylakoid membrane, and assembly with other components of PSII; maturation of the D1 protein via carboxy-terminal processing of pre-D1 and reassembly of PSII complexes (Fig. 1.7) (Nishiyama et al. 2006).

Under strong light, ROS are abundantly produced. The effects of ROS on the repair of PSII were studies in *Synechocystis* (Nishiyama et al. 2001, Nishiyama et al. 2004). When intracellular levels of ROS were increased by addition of H_2O_2 to cell cultures or by inactivation of *katG* and *tpx* genes for catalase and thioredoxin peroxidase, respectively, the extent of photoinhibition of PSII was enhanced via inhibition of the repair of PSII, in particular, of the D1 protein synthesis. Under these conditions, not only the synthesis of the D1 protein but also the synthesis of almost all thylakoid proteins was suppressed. (Nishiyama et al. 2001). Similarly, increases in intracellular $^1O_2$ by addition of photosensitizers, such as rose bengal and ethyl eosin, elevated the extent of photoinhibition of PSII by inhibiting the repair of PSII with suppression of the synthesis of the D1 protein (Nishiyama et al. 2004).

The synthesis of the mature D1 protein consists of several steps, including transcription of *psbA* gene, translation of *psbA* mRNA, insertion of pre-D1, and processing of pre-D1. Analysis of polysomes in *Synechocystis* revealed that ROS inhibit the synthesis of the D1 protein *de novo* at the elongation step of translation (Fig. 1.8) (Nishiyama et al. 2001, Nishiyama et al. 2004). Furthermore, elongation factors EF-G and EF-Tu were identified as the targets of ROS within the translational machinery of *Synechocystis*. EF-G is inactivated via

the oxidation, by ROS, of two specific cysteine residues, with subsequent formation of an intramolecular disulfide bond (Kojima et al. 2007, Kojima et al. 2009), while EF-Tu is inactivated via oxidation of the single cysteine residue (Cys82) with subsequent formation of an intermolecular disulfide bond and sulfenic acid (Yutthanasirikul et al. 2016). The particular sensitivity of EF-G to ROS has also been demonstrated in *E. coli* (Nagano et al. 2012, Nagano et al. 2015).

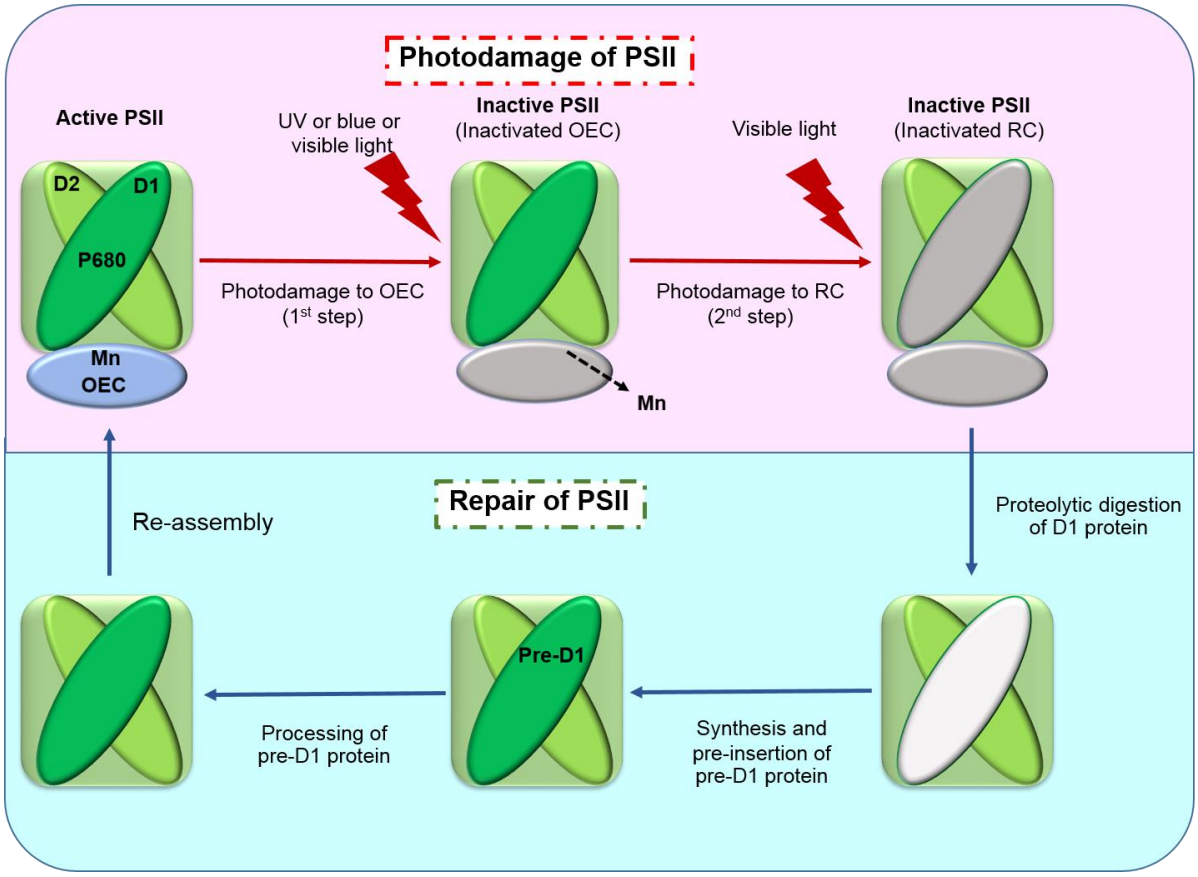

Figure 1.7 The mechanism of photodamage to and repair of PSII. The oxygen-evolving complex (OEC) is inactivated primarily by UV light, blue light and visible light, with resultant release of manganese ions from the PSII complexes. Subsequently, the reaction center (RC) is inactivated by light absorbed by chlorophylls. The repair of photodamaged PSII occurs in the multiple sequential steps. The damaged D1 protein undergoes proteolytic degradation. Then, newly synthesized pre-D1 is inserted into thylakoid membranes and is assembled with other components of PSII. Pre-D1 is processed at the carboxy-terminus to yield mature D1 protein and PSII is reactivated. Adapted from (Takahashi and Murata 2008).

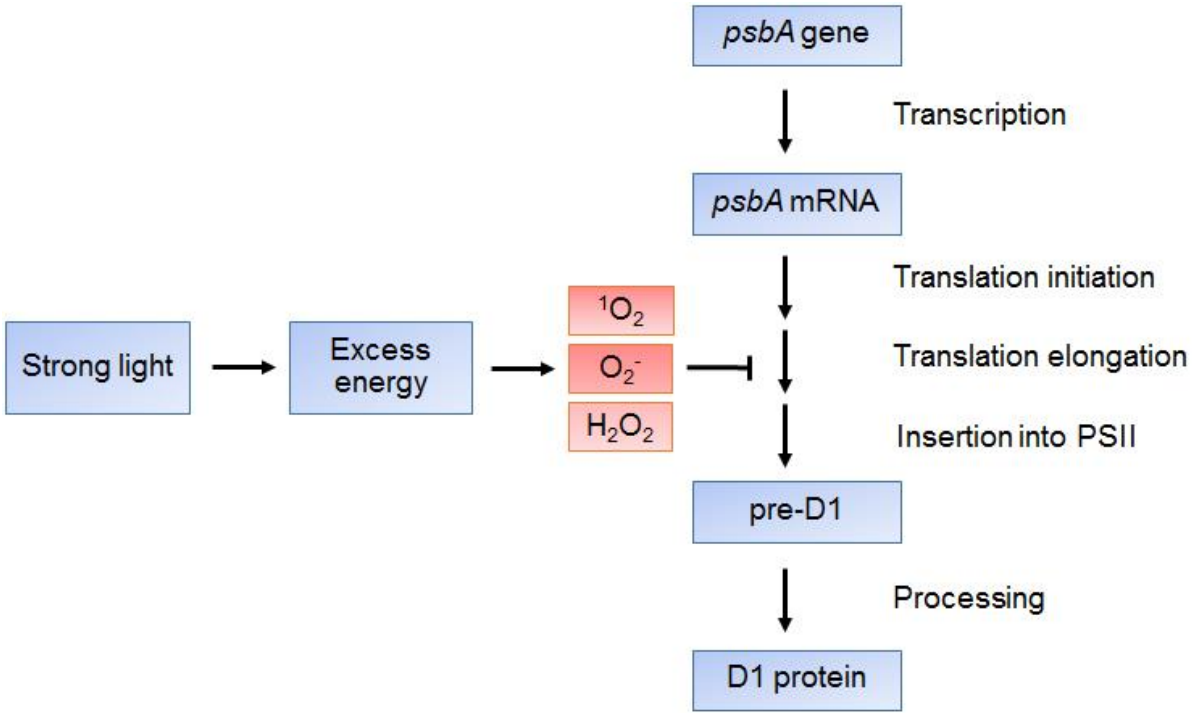

Figure 1.8 A model for the action of ROS in the photoinhibition of PSII. Under strong light, ROS, such as $^1O_2$, O_2^- and H_2O_2, are abundantly produced by the photosynthetic machinery. These ROS suppress the synthesis of proteins, in particular, of the D1 protein, which is a main component of PSII complex. The synthesis of the mature D1 protein consists of several steps, including transcription of *psbA* gene, translation of *psbA* mRNA, insertion of pre-D1 and processing of pre-D1. ROS inhibit the translational elongation step primarily. Adapted from (Nishiyama et al. 2006).

1.3 Reactive oxygen species (ROS)

ROS are generated from the reduction of molecular oxygen in various metabolic processes, including respiration and photosynthesis, as is common in all aerobic organisms. ROS are produced within several organelles, such as chloroplasts, plasma membranes, mitochondria, apoplasts, and nuclei (Gill and Tuteja 2010, Sandalio et al. 2013). In general, ROS also function as signal molecules in many processes such as pathogen defense, systemic signaling and oxidative stress response (Mittler 2002).

In plants, under normal growth conditions, intracellular levels of ROS are maintained low: the concentration of O_2^- is about 240 μM and that of H_2O_2 is about 0.5 μM in chloroplasts (Polle 2001). However, under abiotic stress, the production of ROS is highly accelerated and the levels of O_2^- can rise to 720 μM and those of H_2O_2 can also rise to 5-15 μM (Gill et al. 2015).

Under photo-oxidative stress, excess ROS are generated from the photosynthetic machinery during the transfer of excitation energy and transport of electrons. ROS are produced in various forms, that include free radical molecules, such as OH•, O_2^-, alkoxyl (RO•) and peroxyl (ROO•); and non-radical molecules, such as H_2O_2 and $^1O_2$ (Fig. 1.9) (Gill et al. 2015).

ROS are highly toxic in overall cellular homeostasis by damaging thylakoid membranes via lipid peroxidation of unsaturated fatty acids and also by inactivating the protein-synthetic machinery (Nishiyama et al. 2011). Furthermore, treatment of *Synechocystis* cells with methyl viologen, an inducer of O_2^-, enhanced the photoinhibition by inhibiting the repair of PSII without affecting photodamage to PSII (Nishiyama et al. 2001).

Each species of ROS has different characteristics in terms of localization, toxicity, half-life, and targets. $^1O_2$ is arisen from photosynthetic transfer of excitation energy (Asada 1999). O_2^- is formed by the reduction of oxygen on the acceptor side of photosystem I (PSI)

via Mehler reaction, and it is rapidly converted to H_2O_2, which is further converted to a highly toxic radical, OH• (Fig. 1.10) (Fujii et al. 1990, Asada 1999). OH• is the most harmful radical but there are no enzymes that can scavenge this radical. The potential way to reduce the oxidative damage by OH• is to regulate the reactions that induce its production (Apel and Hirt 2004).

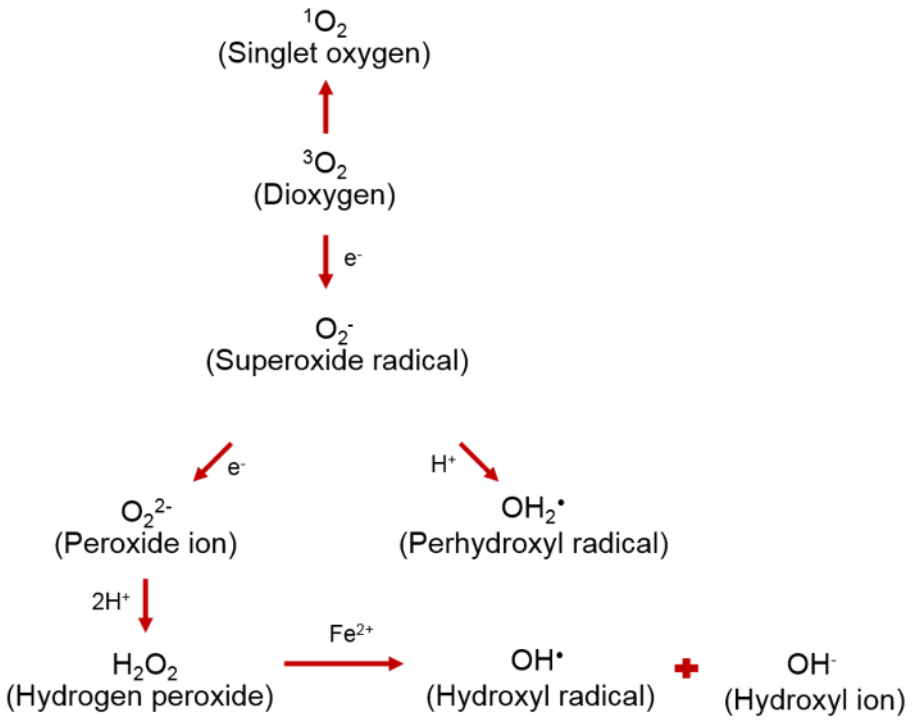

Figure 1.9 Generation of ROS during energy transfer and electron transport. Adapted from (Apel and Hirt 2004).

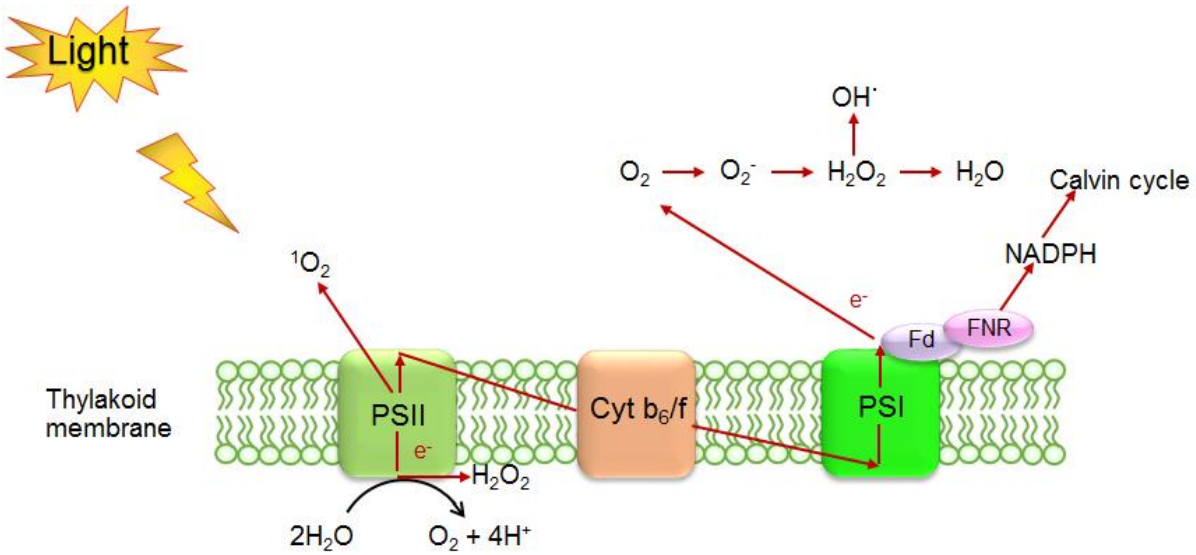

Figure 1.10 A simplified model for the production of ROS from the photosynthetic machinery. $^1O_2$ is produced as a result of the photosynthetic transfer of excitation energy, while O_2^-, H_2O_2, and OH• are produced as a result of the photosynthetic transport of electrons.

1.3.1 Singlet oxygen ($^1O_2$)

$^1O_2$ is mainly produced in the photosynthetic machinery, in particular, in PSII. $^1O_2$ is generated from a result of the transfer of excitation energy from the triplet state of chlorophylls (^{3}Chl) to the triplet ground state of oxygen molecules ($^3O_2$) (Asada 1999, Das and Roychoudhury 2014). The excitation energy to generate $^1O_2$ from $^3O_2$ is 94 kJ mol^{-1} (Wilkinson et al. 1995).

$$\text{Chl} \rightarrow {}^3\text{Chl}$$

$$^3\text{Chl} + {}^3O_2 \rightarrow \text{Chl} + {}^1O_2$$

$^1O_2$ has a relatively short life time, about 3 μs, and it can diffuse up to 100 nm in a single cell (Hatz et al. 2007). Due to the short distance of migration, it is believed that it damages to proteins, pigments, lipids, and nucleic acids near the production site (Triantaphylides and Havaux 2009).

$^1O_2$ can be generated by photosensitizers, which include rose bengal, fluorescein, erythrosine B, eosin and methylene blue (Kochevar and Redmond 2000, DeRosa and Crutchley 2002). 10^3-10^5 molecules of $^1O_2$ are produced by one photosensitizer before degradation of the photosensitizer via photobleaching (DeRosa and Crutchley 2002).

In photosynthetic organisms, $^1O_2$ is detoxified by non-enzymatic antioxidants, such as α-tocopherol (Inoue et al. 2011), plastoquinone and proline (Krieger-Liszkay et al. 2008), and zeaxanthin and echinenone (Kusama et al. 2015).

1.3.2 Superoxide (O_2^-)

O_2^- is mainly generated by one-electron reduction of O_2 molecule during the linear electron transport at the exit of PSI (Fujii et al. 1990, Asada 1999). O_2^- is moderately reactive with a short life time of 2-4 μs and a migration distance of 30 nm. It is a negatively charged molecule, which cannot diffuse across biological membranes so that O_2^- damages biomolecules

at the site where it is generated (Das and Roychoudhury 2014). O_2^- does not only cause toxic effects itself but also generates highly reactive OH•. O_2^- generates ferric ion (Fe^{2+}) from ferrous ion (Fe^{3+}), and produces H_2O_2 via disproportion or dismutation. H_2O_2 is an intermediate to produce the highly reactive OH• via Fenton reaction (Halliwell and Gutteridge 1986). These two reactions that generate Fe^{2+} and OH• are collectively known as Haber–Weiss reaction (Haber and Weiss 1932).

$$O_2^- + Fe^{3+} \rightarrow {}^1O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^- \qquad \text{(Fenton reaction)}$$

Methyl viologen is often used to facilitate the production of O_2^-. It accepts electrons from PSI and transfers electrons to oxygen molecules, leading to the production of O_2^-. Photosynthetic organisms can effectively detoxify O_2^- by superoxide dismutase (SOD) (Perry et al. 2010, Gill et al. 2015).

1.3.3 Hydrogen peroxide (H_2O_2)

H_2O_2 is one of the most abundant ROS. H_2O_2 is relatively stable, non-radical molecule with a long half-life of 1 ms. Thus, it is able to diffuse through biological membranes and traverses for a long distance, for example, 1 μm. H_2O_2 is formed either non-enzymatically by two-electron reduction of oxygen molecule, or enzymatically by dismutaion of O_2^- by SOD (Özyürek et al. 2010, Das and Roychoudhury 2014).

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

$$2O_2^- + e^- + 2H^+ \rightarrow H_2O_2$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 damages various biological molecules, such as proteins and membrane lipids. Moreover, it can be a substrate to form a highly active radical OH• in the presence of reduced metal ions, such as Fe^{2+} and Cu^+. H_2O_2 can be detoxified either by non-enzymatic antioxidants,

such as flavonoids and reduced glutathione, or by ROS-scavenging enzymes, such as catalase and peroxidase (Imlay 2008, Das and Roychoudhury 2014).

1.3.4 Hydroxyl radical (OH•)

OH• is the most reactive and harmful ROS. Its half-life is about 1 μm and is able to migrate up to 1 nm. OH• is formed by Fenton reaction as described above (Das and Roychoudhury 2014, Gligorovski et al. 2015).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

Due to its highly reactivity, OH• can rapidly oxidize DNA, leading to oligonucleotide strand breaks and DNA-protein cross-links (Cadet et al. 1999). Moreover, it can extensively damage proteins and lipids. OH• can be detoxified by only non-enzymatic antioxidants, which include carotenoids, flavonoids, reduced glutathione, and proline (Pinto et al. 2003, Das and Roychoudhury 2014).

1.4 Antioxidative systems against ROS in cyanobacteria

In ambient environments, photosynthetic organisms suffer from various abiotic stresses. These stresses can cause oxidative damage via the production of ROS. ROS are greatly harmful to organelles and damage cells, leading to growth suppression and cell mortality. Under oxidative stress, cells rapidly increase levels of antioxidants to defend against ROS. When levels of ROS exceed the capacity of antioxidative systems, organisms severely suffer from oxidative injury (Fig. 1.11). Thus, the rapid and effective elimination of ROS is critical to protect organisms against oxidative damage from ROS.

Photosynthetic organisms, such as cyanobacteria, have long evolved for billion years and developed antioxidative systems to protect themselves from various types of ROS, such as $^1O_2$, O_2^-, H_2O_2, and OH•. Cyanobacteria possess both non-enzymatic and enzymatic antioxidative mechanisms that synergistically protect cells against ROS. The antioxidative systems in *Synechococcus elongatus* PCC 7942 are summarized in Tables 1.2 and 1.3.

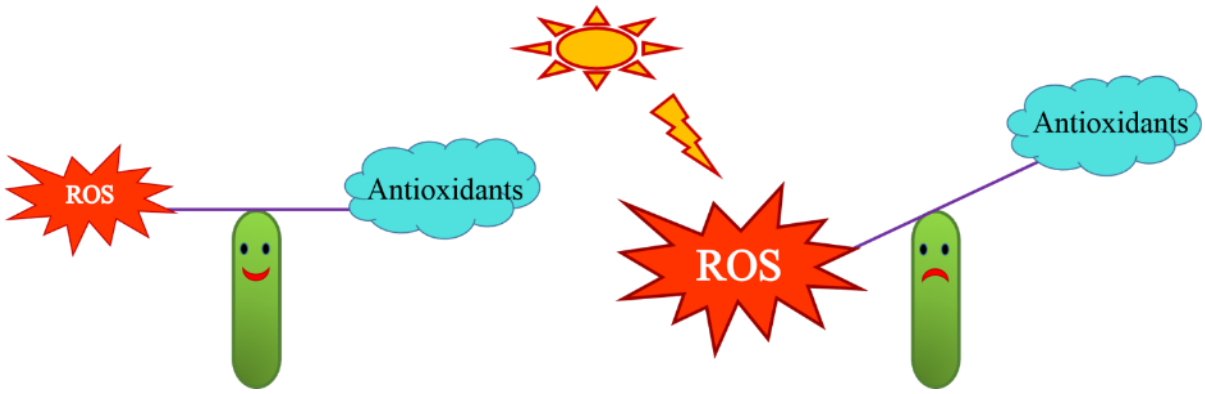

Figure 1.11 The concept of equilibrium and imbalance between ROS and antioxidants.

Table 1.2 Major non-enzymatic antioxidants and antioxidative enzymes in *Synechococcus elongatus* PCC 7942.

Non-enzymatic antioxidants	Function
Reduced Glutathione	Acts as a detoxifying co-substrate for enzymes like peroxidases, GR and GST, and scavenges $^1O_2$, H_2O_2 and OH•
α-tocopherol	Guards against and detoxifies products of membrane LPO, and scavenges $^1O_2$
Carotenoids	Quenches excess energy from the photosystems, LHCs, and scavenge $^1O_2$ and OH•
Flavonoids	Direct scavengers of H_2O_2 and $^1O_2$ and OH•
Proline	Efficient scavenger of OH• and $^1O_2$ and prevent damages due to LPO

Table 1.3 Major enzymatic antioxidants in *Synechococcus elongatus* PCC 7942.

Antioxidative enzymes	Gene name	Enzyme code	Reaction catalyzed
Iron-Superoxide dismutase	*sodB*	1.15.1.1	$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$
Glutathione peroxidase	*SEF0013*	1.11.1.9	$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$
Catalase/peroxidase	*katG*	1.11.1.21	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Thioredoxin peroxidase	*tpxA*	1.11.1.15	$2Trx + H_2O_2 \rightarrow TS\text{-}ST + 2H_2O$

1.4.1 Non-enzymatic antioxidants

Non-enzymatic antioxidants, such as carotenoids, α-tocopherol, ascorbate, and glutathione, are powerful compounds to detoxify ROS (Foyer et al. 1994, Mallick and Mohn 2000, Latifi et al. 2009). These antioxidants are found in various compartments throughout cells. Under oxidative stress, levels of these antioxidants rapidly are increased to protect cells from oxidative damage by preventing the formation of ROS or by scavenging ROS directly.

1.4.1.1 Carotenoids

Carotenoids are the group of pigments that belong to the lipophilic family that play important structural and physiological roles and, in particular, in protecting against oxidative damage (Gruszecki and Strzalka 2005, Reszczynska et al. 2015). Carotenoids are widely present in bacteria, algae, plants, and animals.

In cyanobacteria, the most abundant forms of carotenoids are carotenes, such as β-carotene, and xanthophylls, such as echinenone, synechoxanthin, canthaxanthin, caloxanthin, myxoxanthophyll, nostoxanthin, and zeaxanthin (Zakar et al. 2016). Carotenoids are present both in the cytoplasm and thylakoid membranes. They can absorb light in the wavelength between 400-550 nm and transfer excitation energy to chlorophylls (Koyama et al. 2004). In addition, carotenoids also function as antioxidants to protect the photosynthetic machinery by scavenging $^1O_2$ directly to prevent oxidative damage, quenching excited ^{3}Chl to prevent the formation of $^1O_2$, dissipating excess excitation energy as heat, and suppressing lipid peroxidation (Siefermann-Harms 1987, Young and Frank 1996, Das and Roychoudhury 2014).

Analysis of X-ray crystallography revealed the exact localization of β-carotene in PSII (Loll et al. 2005, Umena et al. 2011). Under low-light conditions, carotenoids assist in harvesting of light via effective absorption of light (Koyama et al. 1996, Bode et al. 2009). However, under strong-light conditions, carotenoids protect PSII from photoinhibition by

dissipating excess energy and by scavenging ROS. To clarify the roles of carotenoids, various carotenoid-deficient mutants were studied. The *crtH/B* and *crtB* mutants of *Synechocystis* sp. PCC 6803 that are deficient in β-carotene and xanthophylls were incapable to form functional PSII reaction center and were extremely sensitive to light, so that they could grow only under heterotrophic conditions (Sozer et al. 2010, Toth et al. 2015). These mutants also showed lower levels of proteins that constitute the reaction center of PSII, such as CP47 and CP43. The *crtO* and *crtR* mutants of *Synechocystis* that are deficient in zeaxanthin and echinenone, respectively, and *crtO*/*R* mutants that lack both of them were sensitive to strong light (Schäfer et al. 2005). Similarly, studies with *crtO* and *crtR* mutants of *Synechocystis* revealed that zeaxanthin and echinenone alleviated the photoinhibition of PSII by enhancement of the repair of PSII, which was achieved by protection of the synthesis of the D1 protein against damage by $^1O_2$ (Kusama et al. 2015). Moreover, orange carotenoid protein (OCP) with hydroxyl echinenone, a derivative of echinenone, was also capable of quenching excess energy as well as scavenging $^1O_2$ (Sedoud et al. 2014). Studies with *ocp* mutant of *Synechocystis* revealed that OCP played a protection role in the repair of PSII by reducing the levels of $^1O_2$ (Kusama et al. 2015).

1.4.1.2 α-Tocopherol

α-Tocopherol belongs to a family of lipophilic antioxidants, which effectively scavenge $^1O_2$, oxygen free radicals, and lipid peroxy radicals (Neely et al. 1988, Di Mascio et al. 1990). Its activity depends on the methylation pattern and the number of methyl groups at the phenolic ring of the polar head structure (Fukuzawa et al. 1982). α-Tocopherol contains three methyl groups, leading to the highest activity among four tocopherol isomers (α, β, γ, δ) (Kamal-Eldin and Appelqvist 1996). One α-tocopherol molecule is capable of scavenging $^1O_2$ up to 120 molecules, as was shown in measurements with resonance energy transfer (Munne-

Bosch 2005). α-Tocopherol is found only in photosynthetic organisms and present both in the envelopes and in the thylakoid membranes of chloroplasts.

Many studies have shown that α-tocopherol acts as an effective antioxidant. The levels of α-tocopherol was up-regulated under low-temperature stress in *Eulgena gracilis* (Ruggeri et al. 1985), under UV-B irradiation in *Chlorella vulgaris* (Malanga and Puntarulo 1995), under salinity and copper stresses in *Anabaena doliolumunder* (Srivastava et al. 2005). The mutants that lack enzymes for tocopherol biosynthesis increased the sensitivity to salt stress in rice (Ouyang et al. 2011) and to oxidative stress in *Arabidopsis* (Semchuk et al. 2009).

In addition, α-tocopherol is associated with the protection of PSII from photoinhibition (Krieger-Liszkay et al. 2008). Examination of an inhibitor of 4-hydroxyphenylpyruvate dioxygenase involved in the biosynthesis of α-tocopherol revealed that α-tocopherol might protect PSII from photoinhibition in *Chlamydomonas* (Trebst et al. 2002, Trebst et al. 2004, Kruk et al. 2005). A mutant of *Arabidopsis* that is deficient in *vte1* for an enzyme involved in α-tocopherol biosynthesis was more sensitive to photoinhibition of PSII than that of wild-type line (Havaux et al. 2005), and this sensitivity was attributed to the inhibition of the repair of PSII (Hakala-Yatkin et al. 2011). In cyanobacteria, the *hpd* mutant of *Synechocystis*, which lacks a key enzyme in α-tocopherol biosynthesis, was highly sensitive to strong light. This sensitivity was not due to acceleration of photodamage to PSII but to inhibition of repair of PSII (Inoue et al. 2011). This study also suggested that α-tocopherol protects the synthesis of the D1 protein from oxidative inhibition (Inoue et al. 2011).

1.4.1.3 Glutathione

Glutathione is a thiol tripeptide (γ-glutamyl-cysteinyl-glycine) that is abundantly present (1-10 mM) both in prokaryotes and eukaryotes (Zhang and Forman 2012, Narainsamy et al. 2016). Glutathione is found mostly in reduced form in almost all cellular compartments,

such as the cytosol, the endoplasmic reticulum, mitochondria, chloroplasts, vacuoles, peroxisomes, and apoplasts (Jimenez et al. 1998). It functions in various physiological processes, including the regulation of sulfate transport, signal transduction, detoxification of xenobiotics, conjugation of metabolites, regulation of enzymatic activity, protection of thiol groups, and defense against ROS-induced oxidative damage (Mullineaux and Rausch 2005). Due to its high reducing potential, glutathione maintains the normal reduced state of cells to prevent oxidative damage. Glutathione is able to directly scavenge $^1O_2$, H_2O_2, and OH• (Gill and Tuteja 2010, Das and Roychoudhury 2014). It is also involved in the regeneration of another antioxidant, ascorbate, via the ascorbate-glutathione cycle (Foyer and Halliwell 1976).

The *gshB* mutant of *Synechocystis* sp. PCC 6803, which is deficient in reduced glutathione due to the lack of an enzyme for glutathione biosynthesis, is highly sensitive to H_2O_2, methyl viologen, and rose bengal. Moreover, this mutant also grows more slowly than wild type under the normal condition and is unable to survive under oxidative stress (Cameron and Pakrasi 2010).

1.4.1.4 Flavonoids

Flavonoids are a large class of secondary metabolites that consist of more than 10,000 different molecules. They are polyphenolic compounds that can be classified on the basis of their two aromatic cycles (A- and B-rings) linked by a heterocycle (C-ring) (Pourcel et al. 2007). Flavonoids are widely distributed in various compartments including chloroplasts and vacuoles. These compounds play important roles as antioxidants to protect cells from oxidative damage by preventing the generation of ROS and reducing the levels of ROS (Agati et al. 2012). Many flavonoid compounds are well-known for their strong scavenging capacity to ROS, such as gallic, chlorogenic, ferulic and vanillic acids, rutin, and quercetin (Karamać et al. 2005). Under various stress conditions, such as drought, metal toxicity, and nutrient deprivation, amounts of

many flavonoid compounds are significantly increased (Winkel-Shirley 2002). For example, under salt stress conditions, polyphenolics and flavonoids are highly accumulated and enhanced antioxidant activity to protect cells from oxidative damage in cyanobacteria (Singh et al. 2014). In addition, the *tt4* and *tt5* mutants of *Arabidopsis*, which is deficient in flavonoids, greatly suffered from oxidative damage causing by high levels of ROS under UV irradiation (Filkowski et al. 2004).

1.4.1.5 Ascorbate

Ascorbate is a most abundant, low-molecular weight, and water-soluble antioxidant that plays a key role in antioxidative systems. It is present in both chloroplasts and the cytosol. Ascorbate is a powerful antioxidant because of its ability to donate electrons to enzymatic and non-enzymatic reactions. Ascorbate is found mainly in the reduced form under normal conditions (Smirnoff and Wheeler 2000). Ascorbate is able to detoxify H_2O_2 via the ascorbate-glutathione cycle (Mallick and Mohn 2000). In cyanobacteria, UV-B induces the production of antioxidants including ascorbate (Kumar et al. 2016). Similarity, light and drought stresses also induced the accumulation of ascorbate in *Picea asperata* seedlings (Yang et al. 2008).

1.4.1.6 Proline

Proline is an osmolyte that plays a role in antioxidative systems. Proline is able to inhibit lipid peroxidation (Mehta and Gaur 1999) and scavenges OH• (Smirnoff and Cumbes 1989) and $^1O_2$ (Alia et al. 2001). Under abiotic stress conditions, the levels of proline significantly increase. In the cyanobacterium *Westiellopsis prolifica*, intracellular levels of proline were elevated under high concentrations of salinity, pesticides, and heavy metals (Fatma et al. 2007). In addition, overexpression of *ArabidopsisP5CS* cDNA in tobacco induced the production of proline and increased tolerance to salt stress (Hmida-Sayari et al. 2005).

1.4.2 ROS-scavenging enzymes

ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase, play critical role in protection of organisms from stress conditions. SOD is the first line of defensive enzymes, which dismutates O_2^- to H_2O_2 and O_2. Catalase and peroxidase scavenge H_2O_2 to H_2O and O_2 (Mallick and Mohn 2000, Latifi et al. 2009). Although OH• is the most harmful radical, there are no enzymes that can scavenge this ROS. A potential way to reduce oxidative damage by OH• is to regulate the reactions that induce the production of OH• by ROS-scavenging enzymes or non-enzymatic antioxidants (Apel and Hirt 2004).

1.4.2.1 Superoxide dismutase

Superoxide dismutase (SOD; EC 1.15.1.1) is a ubiquitous metalloenzyme that effectively functions in the first line of antioxidative systems against oxidative stress. SOD is both a scavenger and a generator of ROS, since this enzyme dismutates O_2^- into H_2O_2 and O_2.

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

In general, SOD is classified into four isoforms according to its metal cofactor; iron (Fe), manganese (Mn), copper/zinc (Cu/Zn), and nickel (Ni) SODs (Fridovich 1997). In addition, there is a cambialistic SOD that exhibits a full activity with either Fe or Mn in its active site (Fe/Mn-SOD). Based on cyanobacterial genome sequences, Fe-SOD and Mn-SOD are the main SOD in cyanobacteria, but it is difficult to distinguish these SOD with their sequences and structures, due to high similarity. Ni-SOD is mostly found in *Synechococcales* and *Chroococcales*, while Cu/Zn-SOD is quite rare and found in *Synechococcales*. Many cyanobacterial species possess multiple SOD genes, which are either the same type or combination of Fe-SOD and Mn-SOD, Mn-SOD and Ni-SOD, and Ni-SOD and Cu/Zn-SOD. However, a primitive marine unicellular *Prochlorococcus* species has only Ni-SOD (Priya et

al. 2007). In addition, Fe-SOD, Mn-SOD, and Cu/Zn-SOD can be distinguished by their sensitivity to potassium cyanide or H_2O_2 in assay of SOD activity (Table 1.3) (Alscher et al. 2002).

Table 1.3 List of SODs and their properties in cyanobacteria.

SOD isozymes	Location	Resistant to	Sensitive to
Fe-SOD	cytosol	KCN	H_2O_2
Mn-SOD	membrane and cytosol	KCN and H_2O_2	-
Cu/Zn-SOD	cytosol	-	KCN and H_2O_2
Ni-SOD	cytosol	-	-

1.4.2.1.1 Fe-SOD

Fe-SOD is the earliest known form of SOD, which might have been initially encoded in plastid genomes and moved to nuclear genomes (Alscher et al. 2002, Scandalios 2002). Fe-SOD has two forms, namely, homodimer and tetramer, in which each monomer contains Fe in the active site. The sequence and structure of Fe-SOD are similar to those of Mn-SOD, suggesting that Fe-SOD and Mn-SOD might share the same ancestor and have co-evolved. However, the specificity of each SOD to the metal is strict. The substitution of iron by manganese in Fe-SOD resulted in extremely low or no activity as dismutase, and vice versa (Lah et al. 1995, Vance and Miller 1998, Renault et al. 2000).

In cyanobacteria, Fe-SOD is a major SOD and present in the cytosol. In contrast, Fe-SOD is not a major SOD in plants, and it is present in the cytosol, chloroplasts, peroxisomes, and apoplasts. Cyanobacterial Fe-SOD consists of 199-229 amino acid residues with molecular masses of 21-25 kDa (Priya et al. 2007). Fe-SOD is sensitive to H_2O_2 but resistant to KCN (Alscher et al. 2002). Many cyanobacterial species maintain multiple SOD, but some cyanobacterial species, such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Arthrospira platensis* MIYE 101, possess only Fe-SOD.

In cyanobacteria, the antioxidative role of Fe-SOD was initially revealed in *Synechococcus elongatus* PCC 7942. A *sodB* mutant line was highly sensitive to light, suggesting a protective role of this SOD under photo-oxidative stress (Herbert et al. 1992). Moreover, cyanobacterial Fe-SOD protected cells from abiotic stresses, such as chilling (Thomas et al. 1999), heavy metal (Choudhary et al. 2007), and salinity (Singh and Kshatriya 2002).

1.4.2.1.2 Mn-SOD

Mn-SOD is widely distributed in prokaryotes and eukaryotes. This SOD is believed to evolve together with Fe-SOD, due to their structural similarity and phylogenetic relations (Priya et al. 2007). Mn-SOD is a homo-dimer or homo-tetramer, which contains one atom of manganese per monomer. The replacement of manganese by iron in Mn-SOD is not possible, even though Mn-SOD and Fe-SOD are very similar to each other (Fridovich 1986, Alscher et al. 2002). The catalytic site of this enzyme is composed by positively charged amino that interacts with negatively charged O_2^-. In plants, Mn-SOD is located in mitochondria and peroxisomes (del Rio et al. 1992).

Cyanobacterial Mn-SOD consists of 200-316 amino acid residues with molecular masses of 22-34 kDa. It is located in thylakoid membranes and the cytosol (Raghavan et al. 2015). Mn-SOD is resistant to both KCN and H_2O_2. In a nitrogen fixing cyanobacterium *Anabaena* PCC 7120, it was reported that Mn-SOD contributed to the protection of PSI and PSII from strong light (Zhao et al. 2007) and also from methyl viologen-mediated oxidative stress (Raghavan et al. 2015).

1.4.2.1.3 Cu/Zn-SOD

In 1938, an unknown protein was isolated from bovine blood and was regarded as a blue copper-storage protein, erythrocuprein (Mann and Keilin 1938). Later McCord and Fridovich found that the protein was able to dismutate or disproportionate superoxide anion and designated it Cu/Zn-SOD (McCord and Fridovich 1969). Cyanobacterial Cu/Zn-SOD is a homodimeric enzyme in which each monomer consists of 174-233 amino acid residues with molecular mass of 16-23 kDa. Each monomer contains a copper and a zinc at the active site, which are bound to a histidine imidazole (Wolfe-Simon et al. 2005, Priya et al. 2007, Abreu

and Cabelli 2010). The monomeric form is also found in plants (Kanematsu and Asada 1989, Schinkel et al. 2001). Copper functions as the redox-active metal, while zinc stabilizes the whole body of the enzyme, confering a wide-range independence of activity to pH (Marino et al. 1995, Abreu and Cabelli 2010). The replacement of copper by other metals led to the inactivation of the enzyme, whereas neither substitution of zinc by Cd, Co, Cu, and Hg nor deletion of zinc significantly decreased the activity of SOD (Bordo et al. 1994, Marino et al. 1995).

Cu/Zn-SOD is a major SOD in plants, but it is rare in cyanobacteria. In plants, this SOD is mainly present in the cytosol, chloroplasts, peroxisomes, apoplasts, and nuclei (Kasai et al. 2006, Kim et al. 2008, Gill et al. 2015), while it is present in the periplasm in cyanobacteria (Chadd et al. 1996). The primary and tertiary structures of Cu/Zn-SOD are different from those of Fe-SOD and Mn-SOD, suggesting that Cu/Zn-SOD might have differentiated and evolved separately from Fe-SOD and Mn-SOD (Smith and Doolittle 1992). Cu/Zn-SOD is inhibited by both KCN and H_2O_2.

1.4.2.1.4 Ni-SOD

In 1996, a cytoplasmic Ni-SOD was first identified in *Streptomyces* species (Youn et al. 1996). Later, this SOD was found in cyanobacteria, *Prochlorococcus marinus* MIT9313, and was identified based on the conserved sequence similarity (Eitinger 2004). Ni-SOD is also found in actinobacteria (Schmidt et al. 2009). Ni-SOD is a hexamer in which monomer consists of 140-163 amino acids with molecular mass of 15-18 kDa. *In silico* analysis of cyanobacterial complete genomes, *Prochlorococcus* possess only Ni-SOD, which might be related to its primitive photosynthetic machinery and small genome size (1.6-2.4 kb) (Priya et al. 2007). Ni-SOD is present in a few groups of cyanobacteria and is conferred to its evolution after differentiation of eukaryotes and substantial diversity among bacteria.

1.4.2.2 Catalase

Catalase (EC 1.11.1.6) is the first antioxidative enzyme that was discovered and characterized. It contains a tetrameric heme and is widely distributed in prokaryotes and eukaryotes. Catalase plays an essential role in controlling levels of ROS by scavenging H_2O_2 to H_2O and O_2 and preventing further reduction of H_2O_2 to highly toxic OH•. Catalase is unique among antioxidative enzymes since it does not require cellular reducing equivalents.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

In addition, some catalases can act as peroxidase (EC 1.11.1.7) and they are called catalase-peroxidase. Catalase-peroxidase exhibits both catalase and peroxidase activities. Catalase and catalase-peroxidase are very different in terms of the structure and active site (Bernroitner et al. 2009). Catalase-peroxidase is found in both prokaryotes and eukaryotes. In cyanobacteria, the gene that encodes bifunctional catalase-peroxidase (KatG) is distributed to about 30% of all cyanobacterial genomes (Bernroitner et al. 2009). KatG is a homodimer or homotretramer, and the molecular mass of monomer is about 80 kDa (Singh et al. 2008). The crystal structure of KatG has been reported in many cyanobacterial species including *Synechococcus elongatus* PCC 7942 (Wada et al. 2002).

A number of studies have shown that catalase protects cells from oxidative damage by ROS. In *Synechocystis* sp. PCC 6803, catalase activity is correlated with the tolerance of cells to oxidative stress (Rady et al. 1994). In addition, the *katG* mutant of *Synechocystis* sp. PCC 6803 exhibited 30 times lower rate of decomposition of H_2O_2 than wild-type cells (Tichy and Vermaas 1999). Overexpression of catalase KatE, derived from *Escherichia coli*, in rice conferred tolerance to salt stress (Nagamiya et al. 2007). In addition, Al-Taweel et al. (2007) revealed that overexpression of a bacterial KatE in tobacco plants protected the repair of PSII with resultant mitigation of photoinhibition of PSII under salt stress conditions. Similarly, the

overexpression of catalase, derived from *Vibrio rumoiensis*, in the cyanobacterium *Synechococcus elongatus* PCC 7942 also demonstrated that catalase protected PSII from photoinhibition by enhancing the repair of PSII via protection of the synthesis of the D1 protein synthesis against oxidative damage by ROS (Jimbo et al. 2013).

1.4.2.3 Glutathione peroxidase

Glutathione peroxidase (GPX) (EC 1.11.1.7) is a large family of multiple isozymes that uses reduced glutathione (GSH) as the electron donor to reduce H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Margis et al. 2008).

$$H_2O_2 + 2GSH \xrightarrow{GPX} GS\text{-}SG + 2H_2O$$

Glutathione peroxidase is found in various compartments of prokaryotes and eukaryotes: in *Arabidopsis*, this enzyme is found in the cytosol, chloroplasts, mitochondria, and the endoplasmic reticulum (Millar et al. 2003). Overexpression of *Chlamydomonas* glutathione peroxidase in tobacco plants enhanced the tolerance to strong light, chilling and salt stresses (Yoshimura et al. 2004). In addition, overexpression of *Synechocystis* glutathione peroxidase in *Arabidopsis* also increased the tolerance to H_2O_2, methyl viologen, chilling, salinity, and drought stresses (Gaber et al. 2006).

1.5 Aim of the present study

As mentioned earlier, ROS are inevitably produced under photo-oxidative stress, mainly from photosynthesis in photosynthetic organisms. ROS are highly toxic and harmful to cells, in particular, to the photosynthetic machinery. Under abiotic stress, such as strong light, salinity and high temperature, ROS are abundantly generated and PSII undergoes photoinhibition. Similarity, treatments of cell culture of *Synechocystis* with photosensitizer, inducing $^1O_2$ production, or with H_2O_2, enhanced the photoinhibition of PSII (Nishiyama et al. 2001, Nishiyama et al. 2004). The cited studies demonstrated that ROS act primarily by inhibiting the repair of PSII during photoinhibition.

In cyanobacteria, as well as in other photosynthetic organisms, SOD and catalase are responsible for the primary defense mechanisms to scavenge O_2^- and H_2O_2, respectively. Overexpression of SOD efficiently reduces levels of O_2^- and, consequently, it can in turn increases levels of H_2O_2 as the product of the scavenging reaction. In other words, overexpression of catalase and peroxidase can reduce levels of only H_2O_2, whereas O_2^- would be still accumulated. Jimbo *et al.* showed that overexpression of a highly active catalase, VktA, protected PSII from photoinhibiton approximately 10% as compare to wild-type cells in *Synechococcus elongatus* PCC 7942 under strong light (Jimbo et al. 2013). Hence, my study aimed to enhance the PSII activity under strong light by reducing intracellular levels of ROS by overexpression of both SOD and catalase in *Synechococcus elongatus* PCC 7942. I hypothesized that overexpression of SOD and catalase together might synergistically mitigate photoinhibition by decreasing overall intracellular levels of ROS including O_2^-, H_2O_2, and OH• (Fig. 1.12).

Recent studies have revealed that ROS inhibit the repair of PSII. Overexpression of catalase *vktA* in *Synechococcus elongatus* PCC 7942 also enhanced the *de novo* synthesis of the D1 protein under strong light and H_2O_2-mediated oxidative stress (Jimbo et al. 2013). Thus,

I also evaluated the synthesis *de novo* of the D1 protein in SOD- and catalase- overexpressing cells under strong light. I found that overexpression of SOD and VktA synergistically alleviated the photoinhibition of PSII by elevating the repair of PSII via the reduction of levels of ROS.

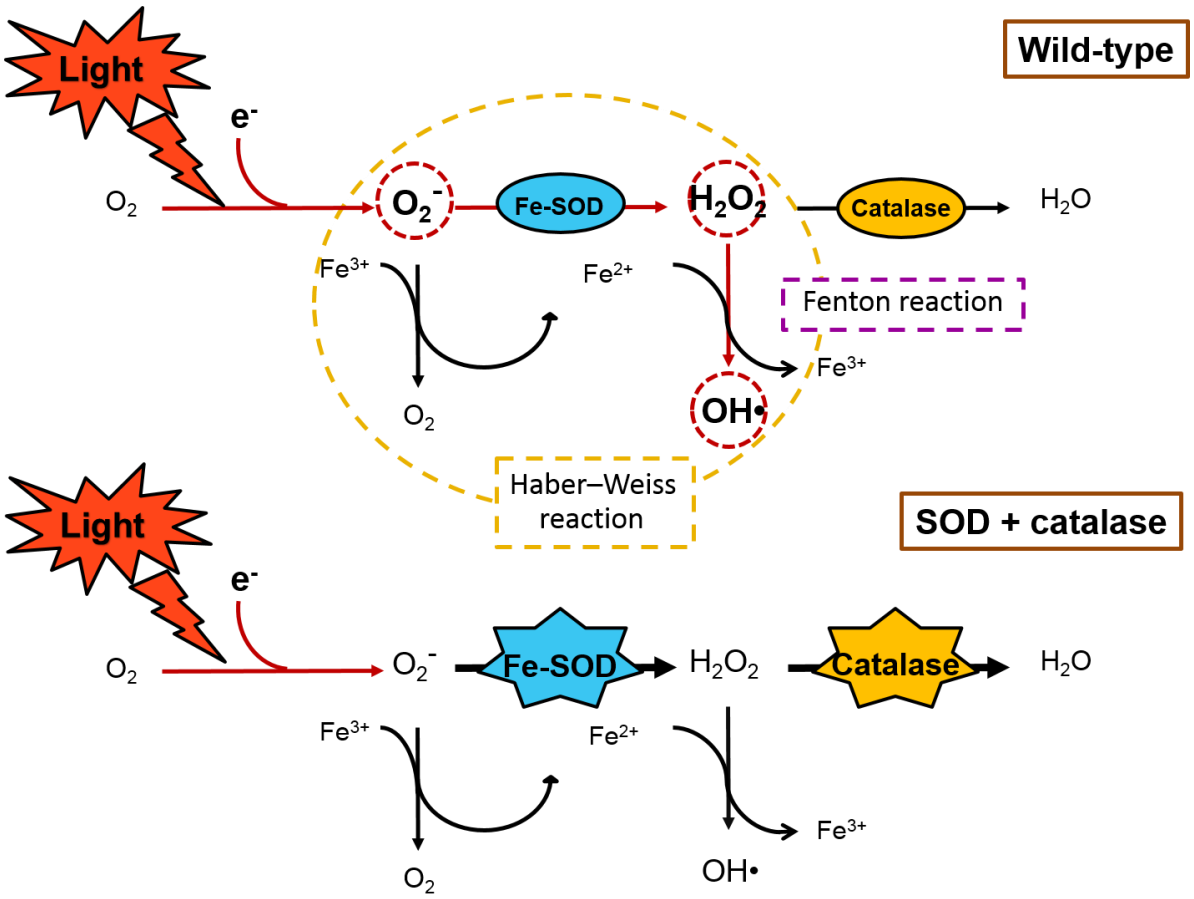

Figure 1.12 A hypothetical model for minimizing the production of ROS in transformed cells that overexpress SOD and catalase under strong light.

Chapter 2

Overexpression of SOD and catalase

in *Synechococcus elongatus* PCC 7942

2.1 Summary

The repair of photosystem II (PSII) under strong light is particularly sensitive to reactive oxygen species (ROS), such as the superoxide radical and hydrogen peroxide, and these ROS are efficiently scavenged by superoxide dismutase (SOD) and catalase, respectively. In the present study, I generated transformants of the cyanobacterium *Synechococcus elongatus* PCC 7942 that overexpressed an iron superoxide dismutase (Fe-SOD) from *Synechocystis* sp. PCC6803; a highly active catalase (VktA) from *Vibrio rumoiensis*; and both enzymes together. Then I examined the sensitivity of PSII to photoinhibition in the three strains. In cells that overexpressed either Fe-SOD or VktA, PSII was more tolerant to strong light than it was in wild-type cells. Moreover, in cells that overexpressed both Fe-SOD and VktA, PSII was even more tolerant to strong light. However, the rate of photodamage to PSII, as monitored in the presence of chloramphenicol, was similar in all three transformant stains and in wild-type cells, suggesting that the overexpression of these ROS-scavenging enzymes might not protect PSII from photodamage but might protect the repair of PSII. Under strong light, intracellular levels of ROS fell significantly, and the synthesis *de novo* of proteins that are required for the repair of PSII, such as the D1 protein, was enhanced. These observations suggest that overexpressed Fe-SOD and VktA might act synergistically to alleviate the photoinhibition of PSII by reducing intracellular levels of ROS, with resultant protection of the repair of PSII from oxidative inhibition.

2.2 Introduction

Photosynthesis is sensitive to various types of environmental stress, and strong light is one of the most significant environmental stressors. Exposure of photosynthetic organisms to strong light often results in the specific inactivation of photosystem II (PSII), and this phenomenon is referred to as photoinhibition of PSII (Powles 1984, Aro et al. 1993). In living cells, PSII is damaged by light and, almost simultaneously, it is repaired by a rapid repair system (Aro et al. 1993, Aro et al. 2005). Thus, photoinhibition results from the imbalance between the rate of the light-induced damage (photodamage) to PSII and the rate of repair of photodamaged PSII (Aro et al. 1993, Aro et al. 2005). Photoinhibition becomes apparent when the rate of photodamage exceeds the rate of repair. In order to understand the nature of photoinhibition, it is necessary to study the processes of photodamage and repair separately, and methods for the separate examination of these processes have been established in cyanobacteria and plants (Gombos et al. 1994, Wada et al. 1994, Moon et al. 1995).

Examination of photodamage and repair separately has revealed several new aspects of the mechanism of photoinhibition, in particular the role of reactive oxygen species (ROS) in the photoinhibition of PSII (Murata et al. 2012, Nishiyama and Murata 2014). When photosynthetic reactions are driven by light energy, ROS are produced as inevitable byproducts by the photosynthetic machinery. The superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•) are produced as a result of the photosynthetic transport of electrons, while singlet oxygen ($^1O_2$) is produced as a result of the photosynthetic transfer of excitation energy via triplet state of chlorophylls (Asada 1999). The production of ROS is greatly stimulated under strong light (Asada 1999). Initially, these ROS, and in particular $^1O_2$, were considered to be the cause of photodamage to PSII (Vass et al. 1992, Hideg et al. 1994, Keren et al. 1997), but recent studies involving examinations of photoinhibition alone demonstrated that ROS act primarily by inhibiting the repair of PSII rather than by accelerating photodamage

to PSII (Nishiyama et al. 2006, 2011, Takahashi and Badger 2011, Murata et al. 2012). For example, in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), photodamage to PSII was not accelerated but the repair of PSII was inhibited by increases in intracellular levels of electron transport-derived ROS, such as O_2^- and H_2O_2. Increases in levels of ROS were achieved by addition of exogenous H_2O_2 and methyl viologen to cells and by suppression of the expression of genes for catalase and thioredoxin peroxidase (Nishiyama et al. 2001); by raising intracellular levels of $^1O_2$ via addition of exogenous photosensitizers, such as rose bengal and ethyl eosin, to cells (Nishiyama et al. 2004); and via suppression of expression of genes for enzymes involved in the synthesis of α-tocopherol (Inoue et al. 2011) and carotenoids, such as zeaxanthin and echinenone (Kusama et al. 2015). By contrast, the repair of PSII was protected by overexpression of the catalase VktA, derived from *Vibrio rumoiensis*, in the cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter referred to as *Synechococcus*) (Jimbo et al. 2013), and also by overexpression of catalase KatE, derived from *Escherichia coli*, in tobacco plants (Al-Taweel et al. 2007). However, the extent of the protection of the repair of PSII by overexpression of catalases in the above-cited studies was limited, and the ROS whose effects must be nullified to protect the repair of PSII remain to be identified.

The photosynthetic transport of electrons generates ROS such as O_2^-, H_2O_2 and OH•. To prevent the generation and accumulation of these harmful ROS, ROS-scavenging enzymes play vital roles. The genomes of *Synechocystis* and *Synechococcus* possess only Fe-SOD (Priya et al. 2007), while many other cyanobacterial species have multiple SODs. Thus, it seems plausible that Fe-SOD might be particularly important among the four metal-SODs. Overexpression of *sodB* from *Synechocystis* in *E. coli* resulted in increased SOD activity and protection of cells from O_2^--mediated oxidative stress (Bhattacharya et al. 2004). However, overexpression of Mn-SOD alone did not diminish the total intracellular levels of ROS because

enhancement of the ability to detoxify O_2^- necessarily raises levels of H_2O_2 (Payton et al. 1997). Overexpression of both SOD and catalase/peroxidase should potentially resolve this problem. Indeed, overexpression of Mn-SOD and catalase in *Bifidobacterium longum* allowed this anaerobic bacterium to tolerate oxygenic growth as a result of an enhanced capacity to decompose O_2^- and H_2O_2 (Zuo et al. 2014). In addition, overexpression of both Cu/Zn-SOD and ascorbate peroxidase in potato and tall fescue plants increased the tolerance of both plants to H_2O_2 and methyl viologen, as well as to high temperature (Tang et al. 2006, Lee et al. 2007).

In the present study, I examined the effects of overexpression of SOD and catalase, separately and together, on the photoinhibition of PSII in *Synechococcus*. I chose Fe-SOD from *Synechocystis* to avoid co-suppression of the expression of the *sodB* gene in the same organism and VktA as the catalase since its catalytic activity is 4.3- and 19-fold higher than that of bovine catalase and KatE of *E. coli*, respectively (Ichise et al. 2000, Yumoto et al. 2000). Under strong light, overexpression of both Fe-SOD and VktA protected PSII from photoinhibition by enhancing the repair of PSII, with decreases in levels of ROS and acceleration of the synthesis of the D1 protein, a core protein in the PSII reaction center.

2.3. Materials and Methods

2.3.1 Cells and culture conditions

Wild-type cells and transformants of *Synechococcus elongatus* PCC 7942 that expressed *sodB* and *vktA* were grown photoautotrophically at 32°C in liquid BG11 medium under light at 70 μmol photons m^{-2} s^{-1}, with aeration by sterile air that contained 1% (v/v) CO_2, as described previously (Kusama et al. 2015). Cells in cultures with an optical density at 730 nm of 1.0 ± 0.1 were used for assays, unless otherwise noted.

To evaluate cell growth under strong light and oxidative stress, cells were grown under standard growth light at 70 μmol photons m^{-2} s^{-1} or strong light at 200 μmol photons m^{-2} s^{-1}. Optical density at 730 nm in cell cultures was monitored at designated times.

2.3.2 Generation of transformants

A DNA fragment containing the *sodA (all0070)* and *sodB* (*slr1516*) genes were amplified from the genomic DNA of *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 with appropriated primers, respectively (Table 2.1). A DNA fragment containing the *vktA* gene and its upstream region (Ichise et al. 2000) from *Vibrio rumoiensis* S-1^T was obtained as described previously (Jimbo et al. 2013). The ends of the DNA fragments were blunted and fragments were inserted into the *Sma*I site of the pAM1044 vector, which was designed to incorporate a gene of interest at a neutral site in the genome of *Synechococcus* and to express the gene under the strong constitutive promoter conII (originally provided by Prof. Susan S. Golden, UC San Diego) (Deshnium et al. 1995). The resultant transformants were designated *sodA*-ox, *sodB*-ox and *vktA*-ox, respectively. For overexpression of both *sodB* and *vktA* together, the plasmid was designed to include a conII promoter in the 5´ upstream regions of both *vktA*

and *sodB* in tandem. The resultant transformant was designated *sodB vktA*-ox. These plasmids were used to transform wild-type *Synechococcus* by homologous recombination.

Table 2.1 Oligonucleotides primers used in this study.

Primer	Sequence, 5'→3'	Purpose	Size (bp)
SodA_F	CCCGGGGCTCAATTTAAGAACAAATA AGAGGC	Vector construction	32
SodA_R	GATATCTTCAACTGAGCGTCACG	Vector construction	23
SodB_F	TTCATATGGCTTACGCACTACCTAAC	Vector construction	26
SodB_R	TTGGATCCGATTTTGCCCAACTCTG	Vector construction	25
VktA_F	GTCGACGATGCAATGAACG	Vector construction	19
VktA_R	GTCGACATTGATCTGACCAG	Vector construction	20
Fw1	CCGCTCGAGCGCTGCTTTCTTGGC	Segregation check	24
Rv1	AACTTCAAGCCGATGTGATC	Segregation check	20

2.3.3 Confirmation of expression of *sodA*, *sodB* and *vktA*

The successful incorporation of the *sodA*, *sodB*, *vktA* and *sodB vktA* genes into all the chromosomal copies of the genome was confirmed by PCR with appropriate primers (Table 2.1). To evaluate the expression of recombinant proteins, cells were disrupted with glass beads and proteins in the soluble fraction were fractionated by SDS-PAGE on a 12% polyacrylamide gel, with subsequent staining with CBB and immunological detection with VktA-specific antibodies, as described previously (Jimbo et al. 2013). To evaluate the activity of Mn-SOD and Fe-SOD, proteins were fractionated at 4°C by PAGE on a 10% non-denaturing gel. After electrophoresis, the gel was soaked for 20 min in a solution of 26 mM *N,N,N',N'*-tetramethylethylenediamine and 25 μM riboflavin and then stained by incubation with 2.5 mM NBT for a further 20 min. The gel was illuminated with white light at 30 μmol photons m^{-2} s^{-1} to initiate the chemical reaction and illumination was continued until transparent bands appeared (Beauchamp and Fridovich 1971).

2.3.4 Assay of the photoinhibition of PSII

Cells were exposed to light at 1,500 μmol photons m^{-2} s^{-1} at 32°C for designated times to induce the photoinhibition of PSII, as described previously (Kusama et al. 2015). For assays of photodamage, chloramphenicol was added to the suspension of cells at a final concentration of 200 mg ml^{-1} just before the onset of illumination. The activity of PSII was measured at 32°C in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone and 1 mM $K_3Fe(CN)_6$ with a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, UK).

2.3.5 Determination of intracellular levels of ROS

Intracellular levels of ROS were measured with the fluorescence indicator carboxy-H_2DCFDA (Invitrogen, Carlsbad, CA, USA), as described previously (Hakkila et al. 2014).

Aliquots of 1 ml of cell suspension were incubated with 15 µM carboxy-H_2DCFDA at 32°C for 1 h in darkness. After cells had been washed and suspended in 0.5 ml of fresh BG11 medium, the suspension was exposed to strong light at 500 µmol photons m^{-2} s^{-1} or to the standard growth light at 70 µmol photons m^{-2} s^{-1} at 32°C for designated times. The fluorescence emitted from the reaction product, 2',7'-dichlorofluorescein, upon excitation at 485 nm was detected at 520 nm with a FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany). All data were normalized by reference to a peak of autofluorescence at 680 nm.

2.3.6 Labeling of proteins *in vivo*

For pulse labeling of proteins, 30 ml of cell culture were incubated at 25°C in light at 1,500 µmol photons m^{-2} s^{-1} for 30 min in the presence of 240 kBq ml^{-1} ^{35}S-labeled methionine plus cysteine (EasyTagTM EXPRE35S ^{35}S; PerkinElmer, Boston, MA, USA), as described previously (Nishiyama et al. 2004). Aliquots of 7 ml of each suspension were withdrawn at designated times for analysis of proteins. Labeling was terminated by the addition of non-radioactive methionine and cysteine to a final concentration of 2 mM each with immediate cooling of samples on ice. Thylakoid membranes were isolated from cells as described previously (Nishiyama et al. 2004), and proteins from thylakoid membranes that corresponded to 10 µg Chl were separated by SDS-PAGE on a 12.5% polyacrylamide gel that contained 6 M urea. Labeled proteins on the gel were visualized with an imaging analyzer (FLA-7000; Fujifilm Life Science, Stamford, CT, USA). Levels of unfractionated labeled proteins in thylakoid membranes were also quantitated by liquid scintillation counting, as described previously (Kojima et al. 2007).

2.4 Results

2.4.1 Overexpression of Mn-SOD and Fe-SOD

The *sodA* gene from *Anabaena* and the *sodB* gene from *Synechocystis*, together with the strong constitutive promoter conII, were inserted into a neutral site in the genome of *Synechococcus* by homologous recombination (Fig. 2.1A). Complete segregation was confirmed by PCR with forward and reverse primers (Table 2.1) that flanked the inserted sequences and genomic DNA as template (Fig. 2.1B). Size of PCR products of wild-type, *sodA*-ox and *sodB*-ox are 1087, 4286 and 4057 bp, respectively.

When proteins in extracts from *sodA*- and *sodB*-transformed cells were separated by SDS-PAGE and stained by Coomassie Brilliant Blue (CBB), as a loading control (Fig. 2.2A). Overexpression of Mn-SOD and Fe-SOD was confirmed by an in-gel assay of SOD. Mn-SOD activity was shown as three clear bands on gel in *sodA* transformed cell. Fe-SOD activity was approximately doubled in *sodB* transformed cell (Fig. 2.2B).

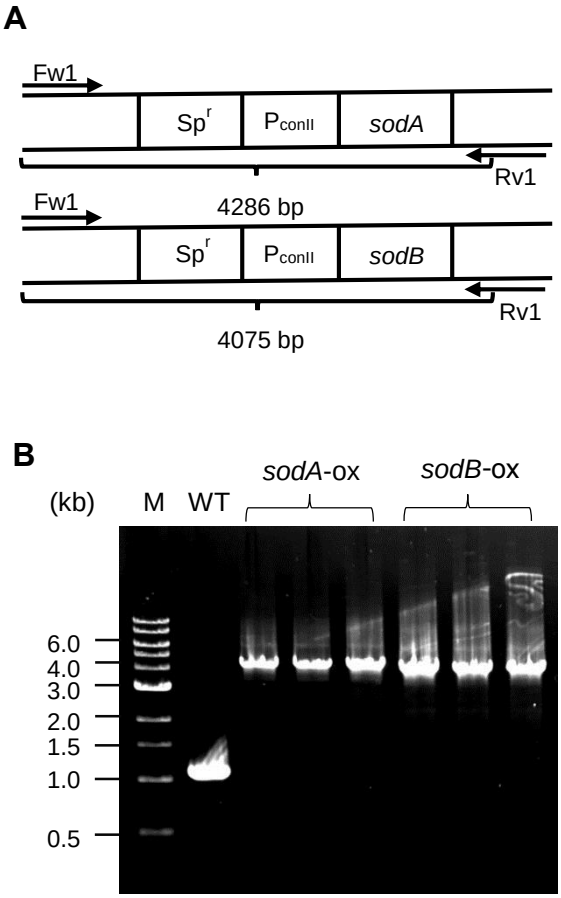

Figure 2.1 Generation of transformants of *Synechococcus* and confirmation of the segregation of genomes. (A) Schematic illustration of the constructs for overexpression of *sodA* and *sodB* in *Synechococcus*. Each gene with the strong constitutive promoter conII (P_{conII}) and a spectinomycin resistance gene cassette (Sp^r) was incorporated into a neutral site of the genome. (B) Analysis by PCR to confirm the complete replacement of the wild-type gene by the appropriate insert. DNA fragments were amplified with specific primers and genomic DNA from wild-type (WT) and transformed cells. Arrows in (A) indicate the positions and directions of forward (Fw1) and reverse (Rv1) primers for PCR. M, molecular size markers.

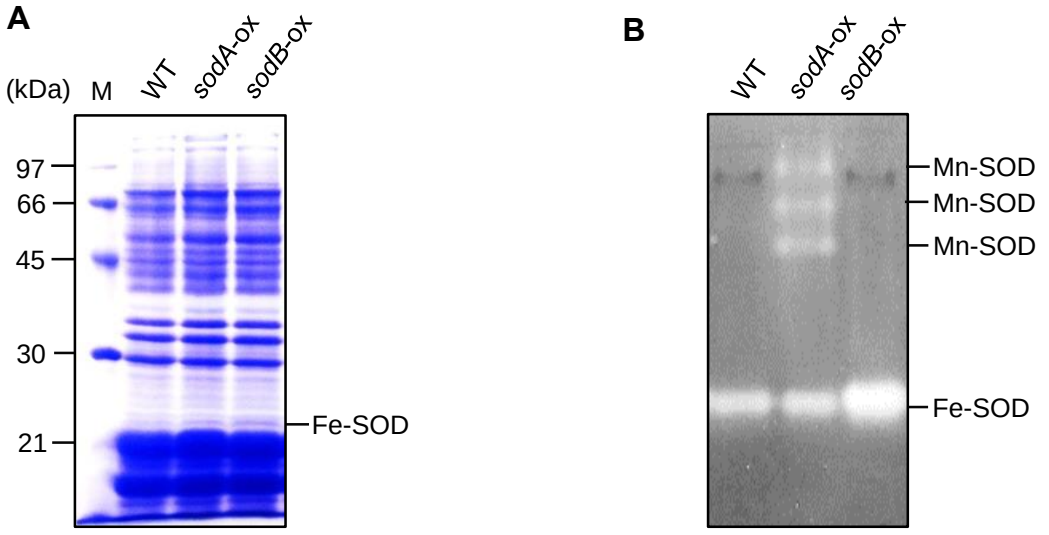

Figure 2.2 Detection of the products of *sodA* and *sodB* in *Synechococcus* cells. Soluble proteins in wild-type and transformed cells. Soluble fractions were isolated from cells, and the proteins in these fractions were analyzed. Proteins (20 mg per lane) were separated by SDS–PAGE on a 12% polyacrylamide gel and stained with CBB as loading control. (B) In-gel assay of the activity of SOD. Proteins (20 mg per lane) were separated on a 10% non-denaturing gel and the activity of SOD was detected as described in the Materials and Methods. M, molecular size markers.

2.4.2 Overexpression of Mn-SOD and Fe-SOD alleviates the photoinhibition of PSII

The effects of overexpression of Mn-SOD and Fe-SOD on photoinhibition of PSII were examined. When wild-type cells and transformed cells were exposed to strong light at 1,500 μmol photons m^{-2} s^{-1} at 32°C, the activity of PSII declined, as is typical during the photoinhibition of PSII (Fig. 2.3A). However, the sensitivity of PSII to strong light differed among wild-type and transformed cells. PSII in *sodA*-ox cells was resistant to strong light as the PSII in *sodB*-ox cells (Fig. 2.3A). Thus, it appeared that the overexpression of either Mn-SOD or Fe-SOD protected PSII from photoinhibition of PSII. By contrast, when cells were exposed to strong light at the same intensity in the presence of chloramphenicol, which blocks the repair of PSII, the activity of PSII declined at the same rate in wild-type and all strains of transformed cells (Fig. 2.3B). These observations suggested that the overexpression of Mn-SOD or Fe-SOD did not protect PSII from photodamage but did protect the repair of PSII, with resultant protection of PSII against photoinhibition.

Methyl viologen facilitates the transfer of electrons from the acceptor side of PSI to molecular oxygen, with resultant production of excess O_2^- within cells. When cells were exposed to strong light at 1,500 μmol photons m^{-2} s^{-1} at 32°C in the presence of 2 μM methyl viologen, the activity of PSII declined more rapidly than that in its absence in all strains of cells. PSII in *sodB*-ox cells was more resistant than the PSII in *sodA*-ox cells (Fig. 2.4A). By contrast, the rate of photodamage to PSII, as measured in the presence of chloramphenicol, did not differ among wild-type and transformed cells (Fig. 2.4B), suggesting that the overexpression of Mn-SOD and Fe-SOD protected the repair of PSII under strong light and in the presence of methyl viologen. It seems that overexpression of Fe-SOD confers more tolerance against photo-oxidative stress than that of Mn-SOD. Thus, I chose Fe-SOD for overexpression and performed the following experiments with Fe-SOD-overexpressing cells.

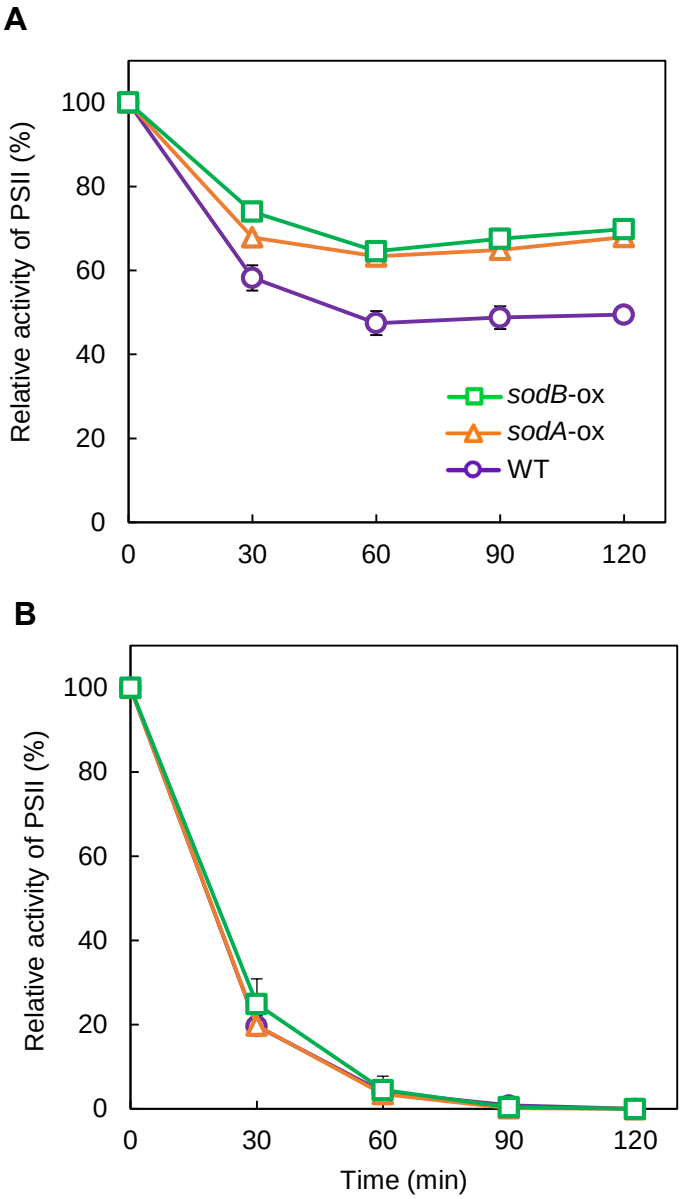

Figure 2.3 Effects of the overexpression of Mn-SOD and Fe-SOD on the photoinhibition of PSII in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 µmol photons m^{-2} s^{-1} with standard aeration in the absence of chloramphenicol (A) and in its presence (B). The activity of PSII was monitored in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. Values are means ± SD (bars) of results from at least three independent experiments. In this and other figures, the absence of a bar indicates that the SD falls within the symbol.

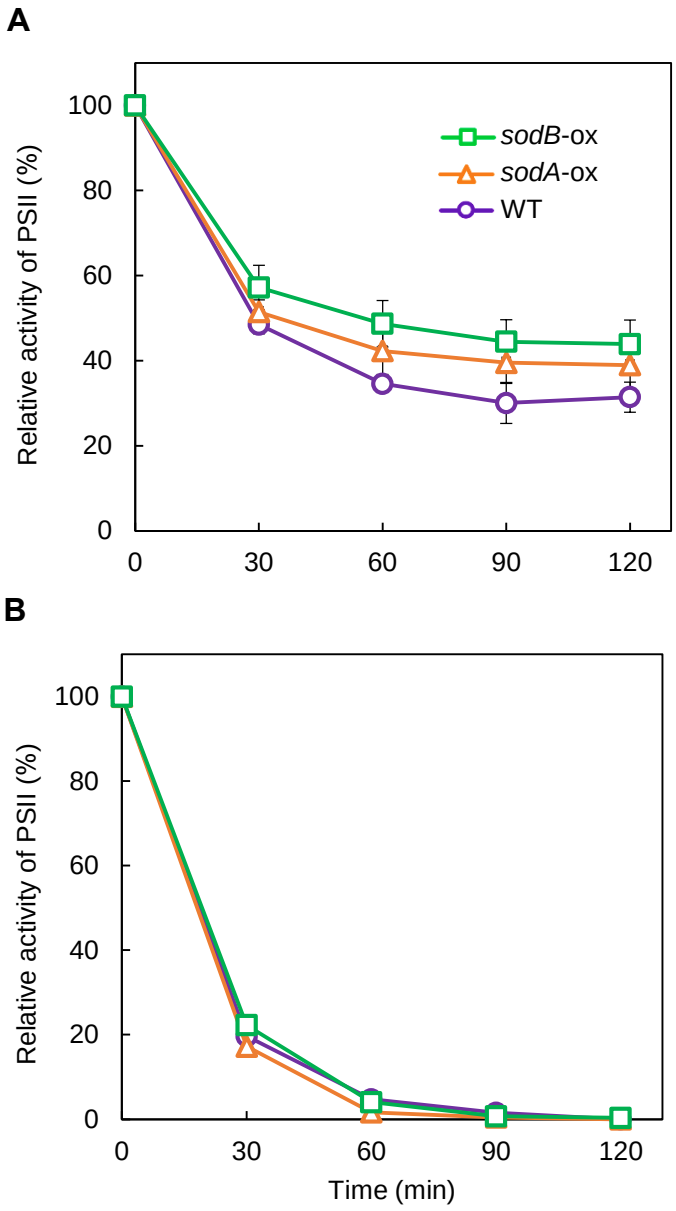

Figure 2.4 Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the photoinhibition of PSII in the presence of 2 mM methyl viologen in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 µmol photons m^{-2} s^{-1} with standard aeration in the absence of chloramphenicol (A) and in its presence (B). Values are means ± SD (bars) of results from at least three independent experiments.

2.4.3 Overexpression of Fe-SOD and VktA

The *vktA* gene from *Vibrio rumoiensis*, together with the strong constitutive promoter conII, was inserted into a neutral site in the genome of *Synechococcus* by homologous recombination (Fig. 2.5A). The resultant transformant was designated *vktA*-ox. The combination of *sodB* and *vktA* genes together, with individual conII promoters, was also inserted into the genome for overexpression of both Fe-SOD and VktA together (Fig. 2.5A). The resultant transformant was designated *sodB vktA*-ox. Complete segregation was confirmed by PCR with forward and reverse primers (Table 2.1) that flanked the inserted sequences and genomic DNA as template (Fig. 2.5B). Size of PCR products of wild-type, *sodB*-ox, *vktA*-ox and *sodB vktA*-ox are 1087, 4057, 5717 and 6656 bp, respectively.

When proteins in extracts from *sodB*- and *sodB vktA*-transformed cells were separated by SDS-PAGE and stained by Coomassie Brilliant Blue (CBB), an intense band was detected at 23 kDa, which corresponds to the molecular mass (22.5 kDa) of Fe-SOD (Fig. 2.6A). In the case of *vktA*- and *sodB vktA*-transformed cells, an intense band was detected at 60 kDa, which corresponds to the molecular mass (57.3 kDa) of VktA (Fig. 2.6). Overexpression of Fe-SOD was confirmed by an in-gel assay of SOD, which showed that the activity of SOD had approximately doubled (Fig. 2.6C). Overexpression of VktA was confirmed immunologically with VktA-specific antibodies (Fig. 2.6D).

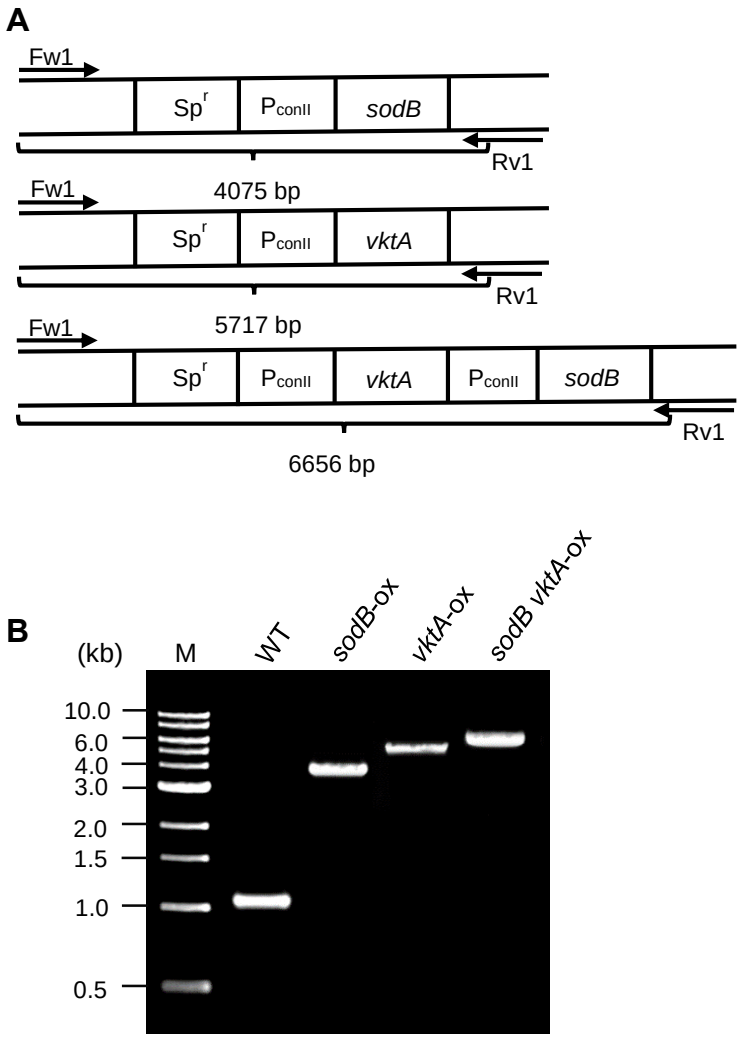

Figure 2.5 Generation of transformants of *Synechococcus* and confirmation of the segregation of genomes. (A) Schematic illustration of the constructs for overexpression of *sodB* and *vktA*, separately and together, in *Synechococcus*. Each gene with the strong constitutive promoter conII (P_{conII}) and a spectinomycin resistance gene cassette (Sp^r) was incorporated into a neutral site of the genome. (B) Analysis by PCR to confirm the complete replacement of the wild-type gene by the appropriate insert. DNA fragments were amplified with specific primers and genomic DNA from wild-type (WT) and transformed cells. Arrows in (A) indicate the positions and directions of forward (Fw1) and reverse (Rv1) primers for PCR. M, molecular size markers.

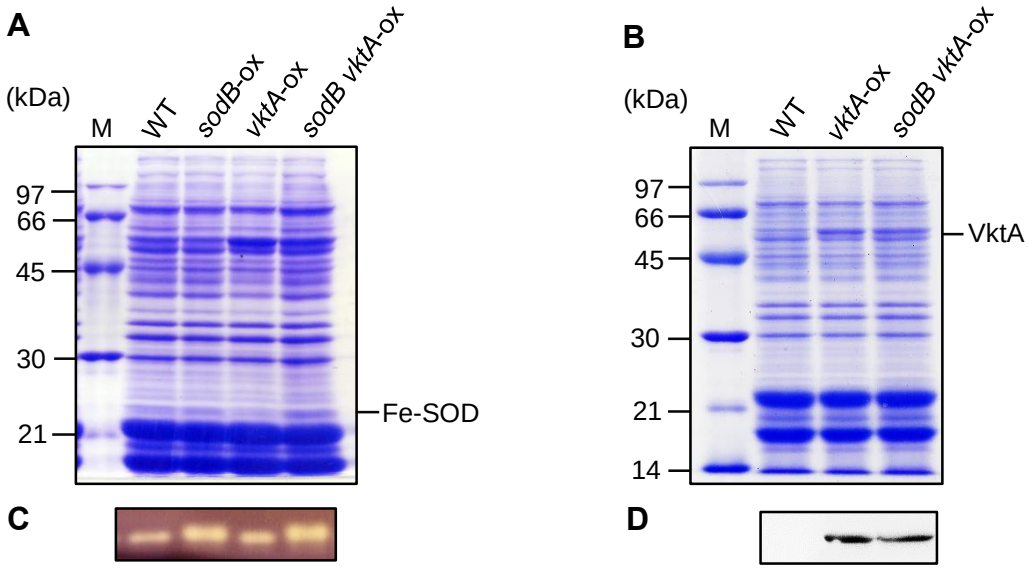

Figure 2.6 Detection of the products of *sodB* and *vktA* in *Synechococcus* cells. (A, B) Soluble proteins in wild-type and transformed cells. Soluble fractions were isolated from cells, and the proteins in these fractions were analyzed. Proteins (20 mg per lane) were separated by SDS–PAGE on a 12% polyacrylamide gel and stained with CBB as loading control. (C) In-gel assay of the activity of SOD. Proteins (20 mg per lane) were separated on a 10% non-denaturing gel and the activity of SOD was detected as described in the Materials and Methods. (D) Detection of VktA. Proteins (20 mg per lane) were separated by SDS–PAGE on a 12% polyacrylamide gel and VktA was detected immunologically. M, molecular size markers.

2.4.4 Overexpression of Fe-SOD and VktA alleviates the photoinhibition of PSII

The effects of overexpression of Fe-SOD and VktA on photoinhibition of PSII were examined. When wild-type cells were exposed to strong light at 1,500 μmol photons m^{-2} s^{-1} at 32°C, the activity of PSII declined, as is typical during the photoinhibition of PSII (Fig. 2.7A). However, the sensitivity of PSII to strong light differed among wild-type and transformed cells. PSII in *vktA*-ox cells was more resistant to strong light than that in wild-type cells; PSII in *sodB*-ox cells was more resistant than that in *vktA*-ox cells; and PSII in *sodB vktA*-ox cells was more resistant than that in *sodB*-ox cells (Fig. 2.7A). Thus, it appeared that the overexpression of either Fe-SOD or VktA protected PSII from photoinhibition of PSII, while the overexpression of both Fe-SOD and VktA together protected PSII from photoinhibition to an even greater extent. By contrast, when cells were exposed to strong light at the same intensity in the presence of chloramphenicol, which blocks the repair of PSII, the activity of PSII declined at the same rate in wild-type and all strains of transformed cells (Fig. 2.7B). These observations suggested that the overexpression of Fe-SOD and VktA did not protect PSII from photodamage but did protect the repair of PSII, with resultant protection of PSII against photoinhibition.

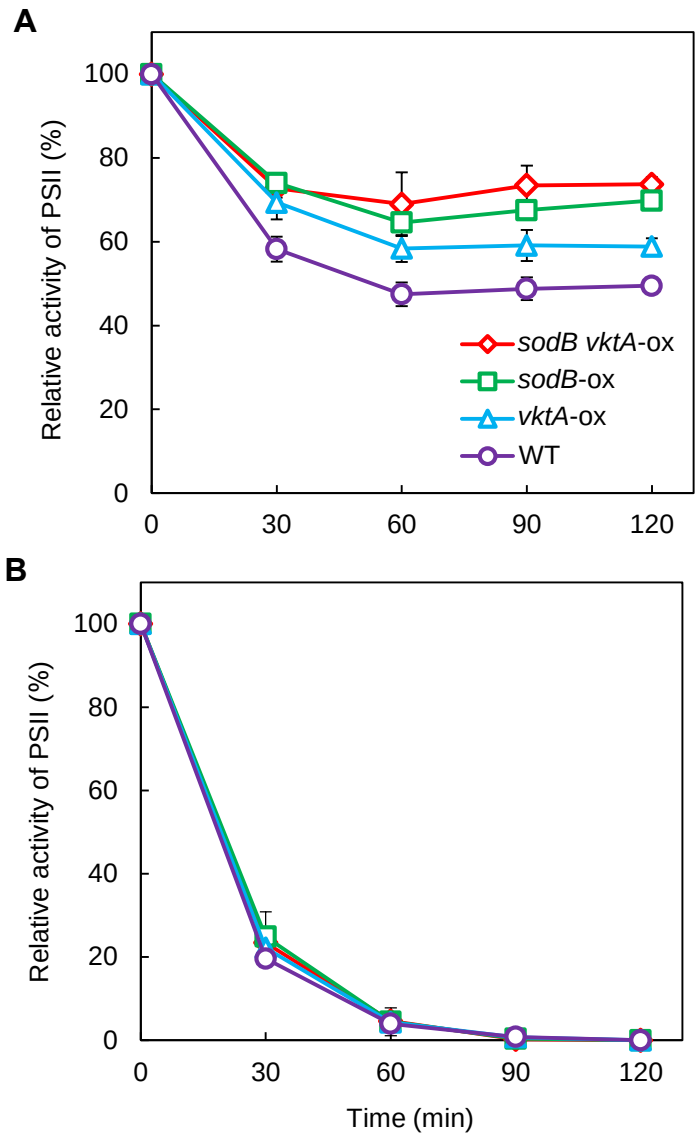

Figure 2.7 Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the photoinhibition of PSII in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 µmol photons m^{-2} s^{-1} with standard aeration aeration in the absence of chloramphenicol (A) and in its presence (B). The activity of PSII was monitored in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. The activities taken as 100% for wild-type, *sodB*-ox, *vktA*-ox and *sodB vktA*-ox cells were 1,600 ± 120, 1,690 ± 80, 1,560 ± 80 and 1,670 ± 150 mmol O_2 mg^{-1} Chl h^{-1}, respectively. Values are means ± SD (bars) of results from at least three independent experiments. In this and other figures, the absence of a bar indicates that the SD falls within the symbol.

2.4.5 Overexpression of Fe-SOD and VktA protects the repair of PSII in the presence of methyl viologen

When cells were exposed to strong light at 1,500 μmol photons m^{-2} s^{-1} at 32°C in the presence of 2 μM methyl viologen, the activity of PSII declined more rapidly than the decline in its absence in all strains of cells. However, the protective effects of overexpression of Fe-SOD and VktA were still apparent (Fig. 2.8A). PSII in *vktA*-ox cells was as resistant to strong light as the PSII in *sodB*-ox cells, and PSII in *sodB vktA*-ox cells was more resistant than the PSII in *sodB*-ox or *vktA*-ox cells (Fig. 2.8A). By contrast, the rate of photodamage to PSII, as measured in the presence of chloramphenicol, did not differ among wild-type and singly or doubly transformed cells (Fig. 2.8B), suggesting that the overexpression of Fe-SOD and VktA protected the repair of PSII in the presence of methyl viologen. Furthermore, rates of photodamage to PSII in the presence of methyl viologen were the same as those in its absence (Fig. 2.7B), suggesting that ROS might not accelerate photodamage to PSII but might inhibit the repair of PSII, as shown previously (Nishiyama et al. 2001).

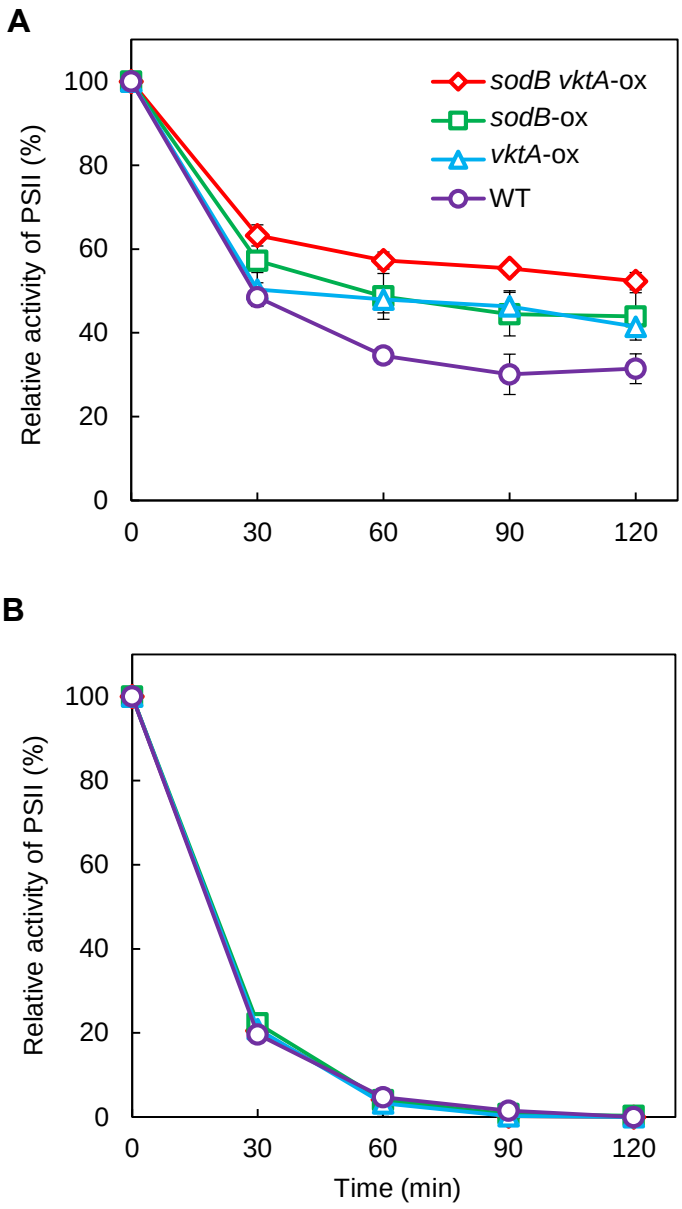

Figure 2.8 Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the photoinhibition of PSII in the presence of 2 mM methyl viologen in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 µmol photons m^{-2} s^{-1} with standard aeration in the absence of chloramphenicol (A) and in its presence (B). Values are means ± SD (bars) of results from at least three independent experiments.

2.4.6 Overexpression of Fe-SOD and VktA depresses intracellular levels of H_2O_2 and related ROS

To examine the effects of overexpression of Fe-SOD and VktA on levels of ROS under strong light, levels of ROS using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) were monitored. Intracellular esterases convert this compound to 2',7'- dichlorodihydrofluorescein, which reacts with ROS that include H_2O_2, hydroxyl and peroxyl radicals, and peroxynitrite but not with O_2^-, with resultant emission of fluorescence (Hakkila et al. 2014). Since this indicator is sensitive to strong light, then, cells were exposed to light at 500 µmol photons m^{-2} s^{-1} at 32°C. In wild-type cells, levels of the above-mentioned ROS rose as illumination was prolonged (Fig. 2.9A). In *sodB*-ox cells, levels of ROS rose at a higher rate than those in wild-type cells (Fig. 2.9A). This phenomenon might have been due to the accumulation of H_2O_2 via the accelerated decomposition of O_2^-. By contrast, the production of ROS was depressed in *vktA*-ox cells and, to a greater extent, in *sodB vktA*-ox cells (Fig. 2.9A). I also observed that the depressed production of ROS in *sodB vktA*-ox cells was even under illumination from a standard growth light at 70 µmol photons m^{-2} s^{-1} for 30 min (Fig. 2.9A). Illumination in the presence of methyl viologen enhanced this trend, with higher production of ROS in wild-type and *sodB*-ox cells and lower production of ROS in *vktA*-ox and *sodB vktA*-ox cells (Fig. 2.9B). Thus, overexpression of Fe-SOD alone did not depress levels of H_2O_2 and related ROS under strong light, whereas overexpression of both Fe-SOD and VktA effectively depressed levels of these ROS. I attempted to monitor changes in intracellular levels of O_2^- using nitro blue tetrazolium (NBT), a specific indicator of O_2^-, under strong light but I failed to detect O_2^- because of the high background due to phycobilisomes around the absorption peak of NBT whose profile changes upon the reaction with O_2^-.

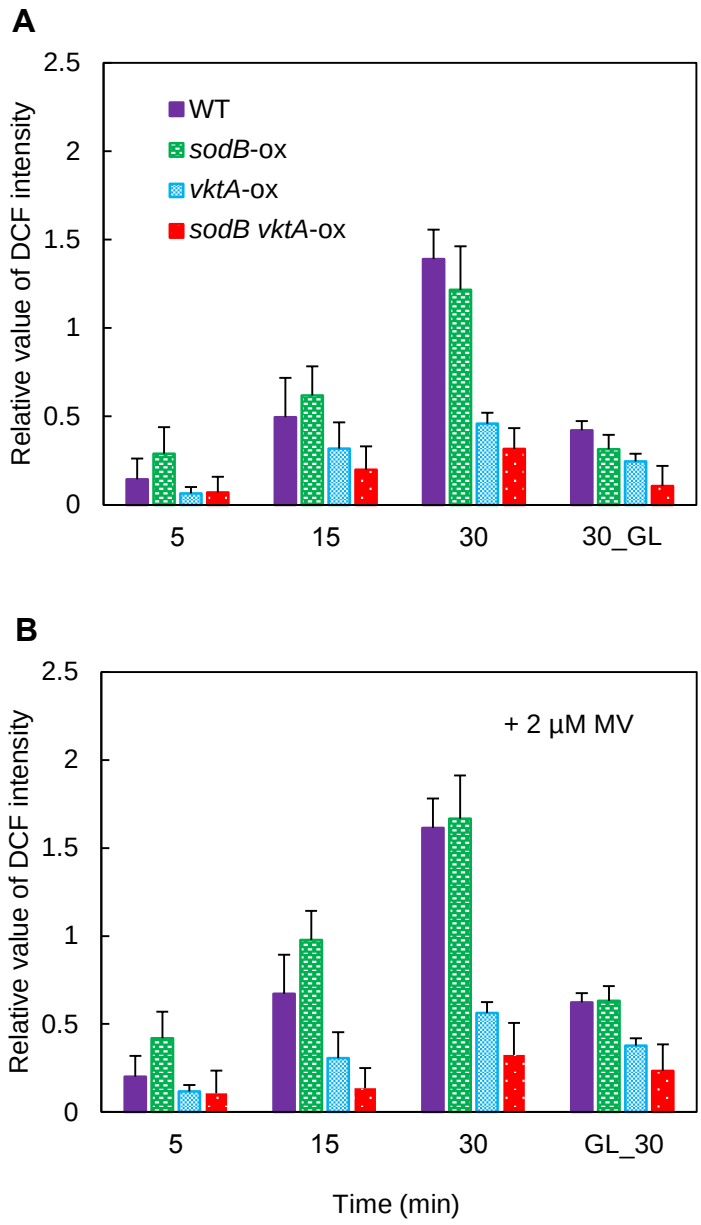

Figure 2.9 Production of H_2O_2 and related ROS during illumination of cells. (A) Cells were illuminated under strong light at 500 µmol photons m^{-2} s^{-1} for the indicated times or under the standard growth light at 70 µmol photons m^{-2} s^{-1} for 30 min (GL_30) in the presence of the fluorescence indicator carboxy-H_2DCFDA. (B) Levels of H_2O_2 and related ROS were monitored under the same conditions as in (A) but in the presence of 2 µM methyl viologen (B). Values are means ± SD (bars) of results from three independent experiments.

2.4.7 Overexpression of Fe-SOD and VktA enhances the synthesis of the D1 protein under strong light

The repair of PSII requires the synthesis *de novo* of the D1 protein, a core protein that constitutes the reaction center of PSII. To examine the effects of overexpression of Fe-SOD and VktA on the synthesis of the D1 protein *de novo* under strong light, cells were exposed to light at 1,500 μmol photons m^{-2} s^{-1} at 25°C in the presence of ^{35}S-labeled methionine and cysteine and monitored radiolabeled proteins in thylakoid membranes on a urea-containing polyacrylamide gel. Proteins on the gel were also stained with CBB as loading controls (Fig. 2.10). The overexpression of Fe-SOD and VktA affected the rate of synthesis of the D1 protein under strong light. Compared to the rate in wild-type cells, the rate of synthesis was slightly higher in *vktA*-ox cells; about 1.4-fold higher in *sodB*-ox cells; and about twice as high in *sodB vktA*-ox cells (Fig. 2.11). Moreover, not only the synthesis of the D1 protein but also the synthesis of many other proteins in thylakoid membranes was enhanced in all three transformed cells (Fig. 2.11). I quantified the entire complement of labeled proteins in thylakoid membranes by liquid-scintillation counting. Levels of proteins that were newly synthesized over the course of 30 min under strong light in *sodB*-ox, *vktA*-ox and *sodB vktA*-ox cells were higher than that in wild-type cells by factors of approximately 1.4, 1.1, and 2, respectively (Fig. 2.12), showing the same trend as that observed in the synthesis *de novo* of the D1 protein. Thus, under strong light, overexpression of both Fe-SOD and VktA accelerated not only the synthesis of the D1 protein but also the synthesis of the other proteins in thylakoid membranes.

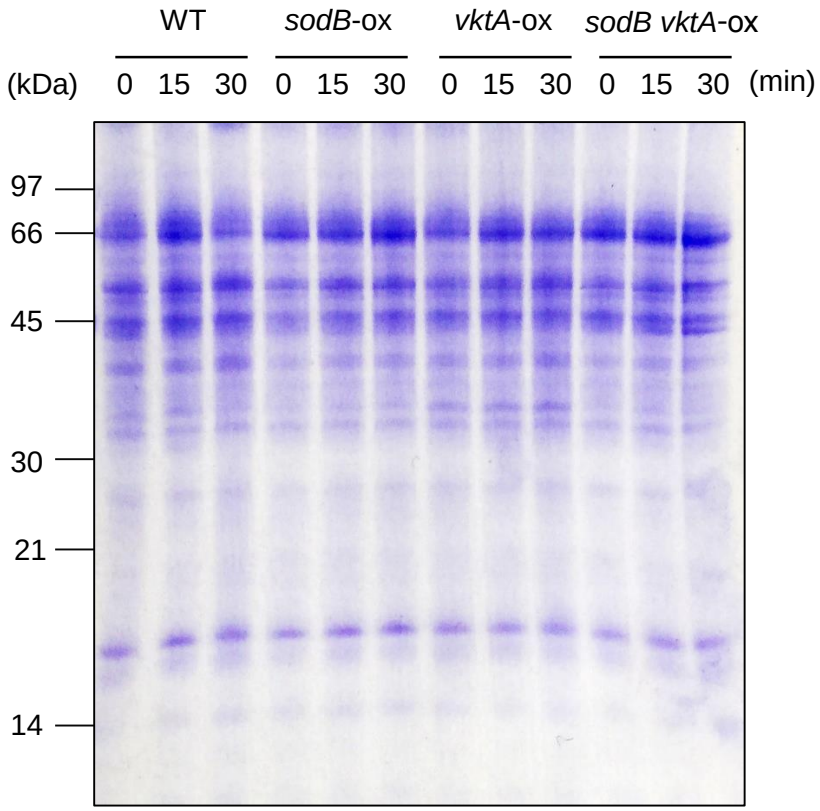

Figure 2.10 Effects of the expression of Fe-SOD, VktA and Fe-SOD plus VktA on the composition of proteins in thylakoid membranes in *Synechococcus* under strong light. Proteins in wild-type (WT) and transformed cells were incubated at 25°C, for the indicated times at 1,500 µmol photons m^{-2} s^{-1}. Proteins in thylakoid membranes that corresponded to 20 µg Chl were separated by SDS-PAGE on a 12% polyacrylamide gel and stained with CBB as loading controls.

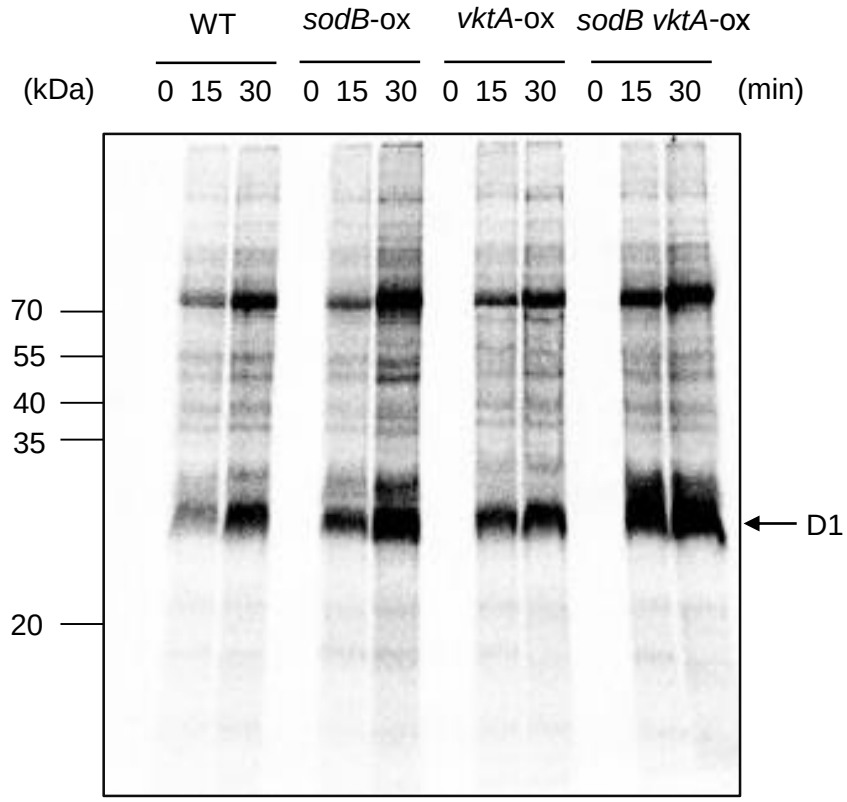

Figure 2.11 Effects of the expression of Fe-SOD, VktA and Fe-SOD plus VktA on the synthesis of the D1 protein *de novo* in *Synechococcus* under strong light. Proteins in wild-type (WT) and transformed cells were pulse-labeled with ^{35}S-labeled methionine plus cysteine during incubation of cells at 25°C, for the indicated times at 1,500 µmol photons m^{-2} s^{-1}. (A) Radiogram of proteins from thylakoid membranes**.**

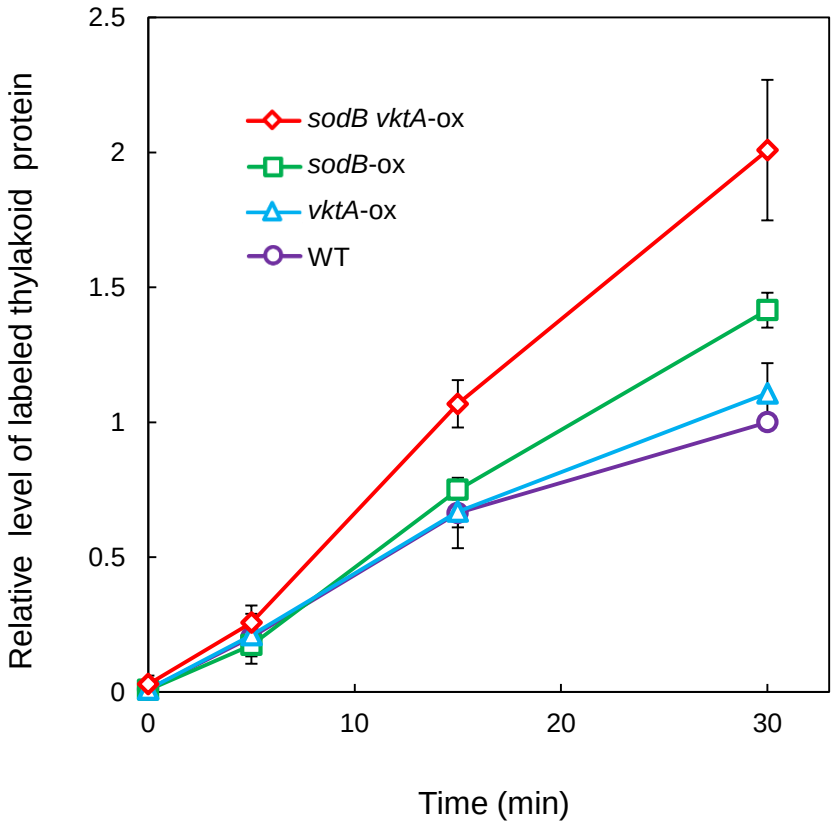

Figure 2.12 Effects of the expression of Fe-SOD, VktA and Fe-SOD plus VktA on the synthesis of the D1 protein *de novo* in *Synechococcus* under strong light. Proteins in wild-type (WT) and transformed cells were pulse-labeled with ^{35}S-labeled methionine plus cysteine during incubation of cells at 25°C, for the indicated times at 1,500 µmol photons m^{-2} s^{-1}. Levels of unfractionated labeled proteins in thylakoid membranes were determined by liquid-scintillation counting. Values are means ± SD (bars) of results from at least three independent experiments.

2.4.8 Effects of overexpression of Fe-SOD and VktA in growth of cells under photo-oxidative stress

To examine the effects of overexpression of Fe-SOD and VktA in growth of cells under photo-oxidative stress, singly and doubly transformed cells were grown under 70 or 200 μmol photons m^{-2} s^{-1}. All three transformed cells and wild-type cells grew at similar rate under light at 70 μmol photons m^{-2} s^{-1} (Fig. 2.13), suggesting that decreases in intracellular levels of ROS might not affect the growth of cells under normal conditions. Unexpectedly, there were no significant differences in the growth rate among the four different strains under strong light at 200 μmol photons m^{-2} s^{-1} (Fig. 2.14). Thus, it seems that not only the protection of PSII from photoinhibition but also some other factors might be required for the tolerance of cell growth to strong light. In other words, cell growth might be determined by multiple factors. Furthermore, I monitored the growth of cells on solid medium in the presence of methyl viologen (Fig. 2.15). Wild-type cells were unable to survive in the presence of 2 μM methyl viologen, but all transformed cells grew. In the presence of 5 μM methyl viologen, a severer oxidizing condition, *sodB*-ox and *sodB vktA*-ox cells survived, but *vktA*-ox cells died. Under this condition, *sodB vktA*-ox cells grew slightly better than *sodB*-ox cells. Since methyl viologen induces the production of O_2^-, *vktA*-ox cells might not have been able to detoxify O_2^-. In contrast, *sodB*-ox cells with overexpressed Fe-SOD might have been able to detoxify O_2^- and survive under the oxidative conditions. In addition, growth of cells in liquid medium in the presence of 1.5 μM methyl viologen also conferred the similar result as that on solid medium (Fig. 2.16). Under this condition, *sodB vktA*-ox cells grew far better than wild-type cells in the presence of methyl viologen-mediated oxidative stress. This results suggested that overexpression of Fe-SOD and VktA increased the tolerance of PSII to methyl viologen-mediated oxidative stress in *Synechococcus*.

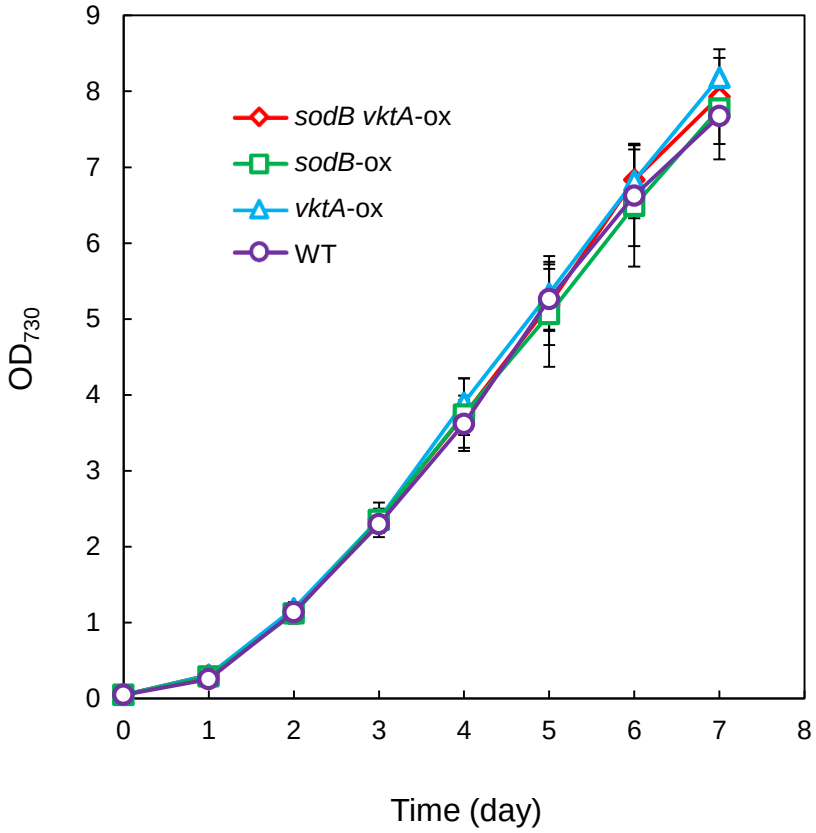

Figure 2.13 Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the growth curve in *Synechococcus*. Wild-type (WT) an d transformed cells were grown under standard growth light 70 µmol photons m^{-2} s^{-1} for 7 days, then measured absorbance of cells at 730 nm. Values are means ± SE (bars) of results from at least three independent experiments.

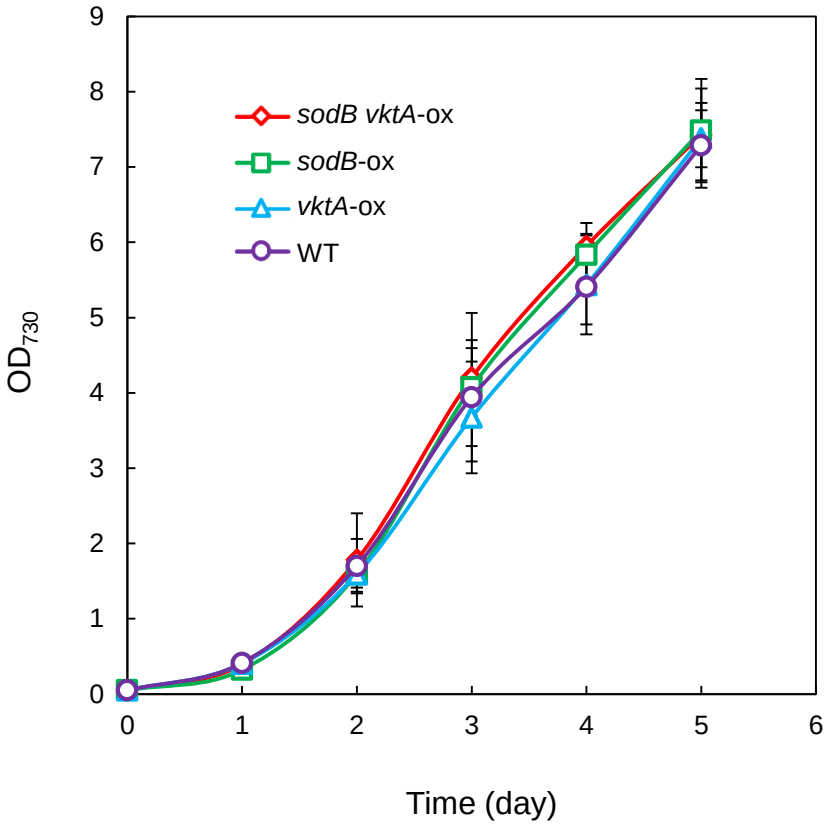

Figure 2.14 Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the on the growth curve in *Synechococcus*. Wild-type (WT) and transformed cells were grown under strong light 200 µmol photons m^{-2} s^{-1}. Growth of cells was monitored in terms of optical density at 730 nm. Values are means ± SE (bars) of results from at least three independent experiments.

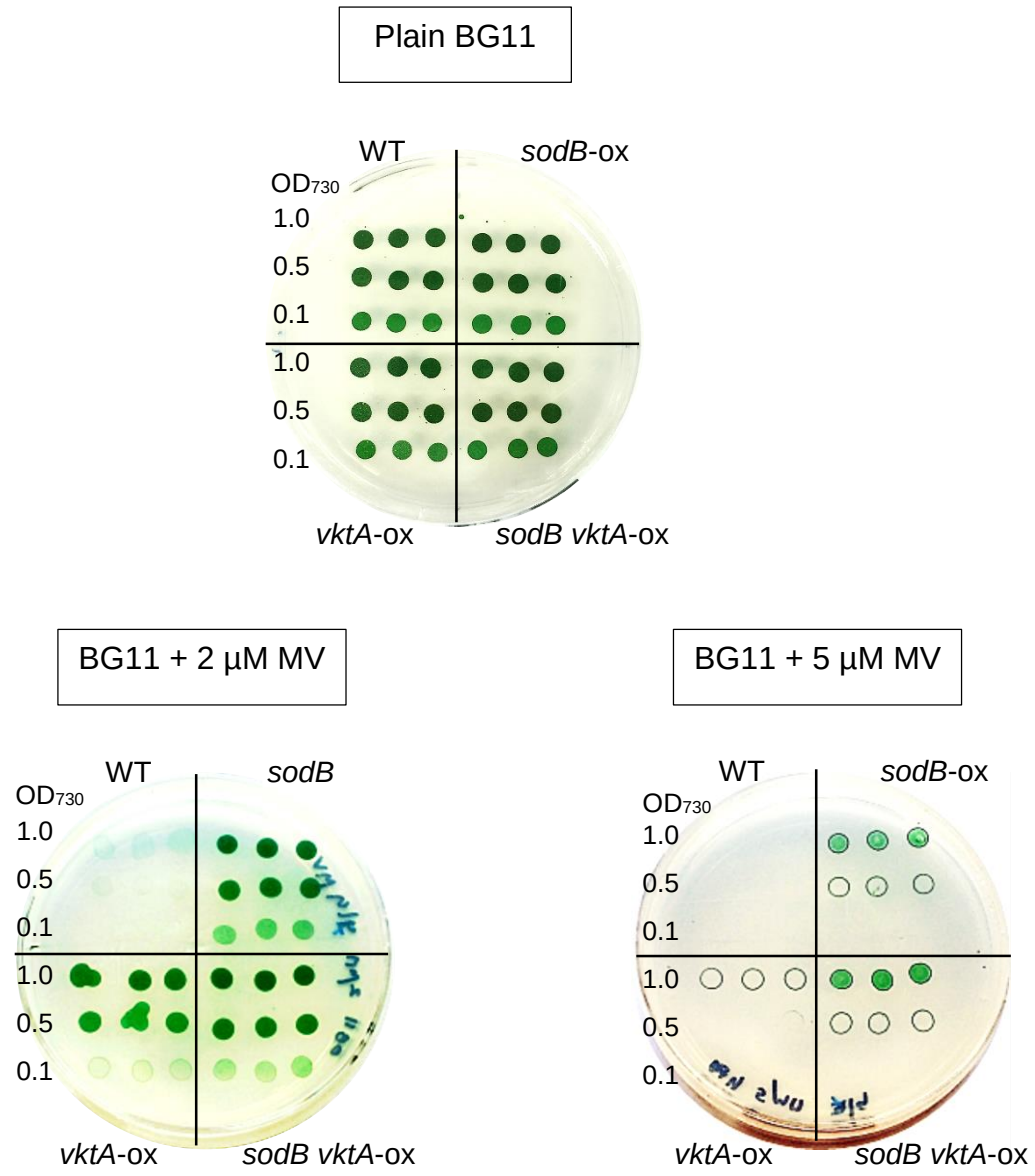

Figure 2.15 Effects of the overexpression of Fe-SOD, VktA, and Fe-SOD plus VktA on the growth under oxidative stress in *Synechococcus*. 5 µl of wild-type (WT) and transformed cell cultures at the indicated optical density at 730 nm were grown in the absence and presence of 2 µM and 5 µM methyl viologen under light at 30 µmol photons m^{-2} s^{-1}. The photos were taken after 7 days.

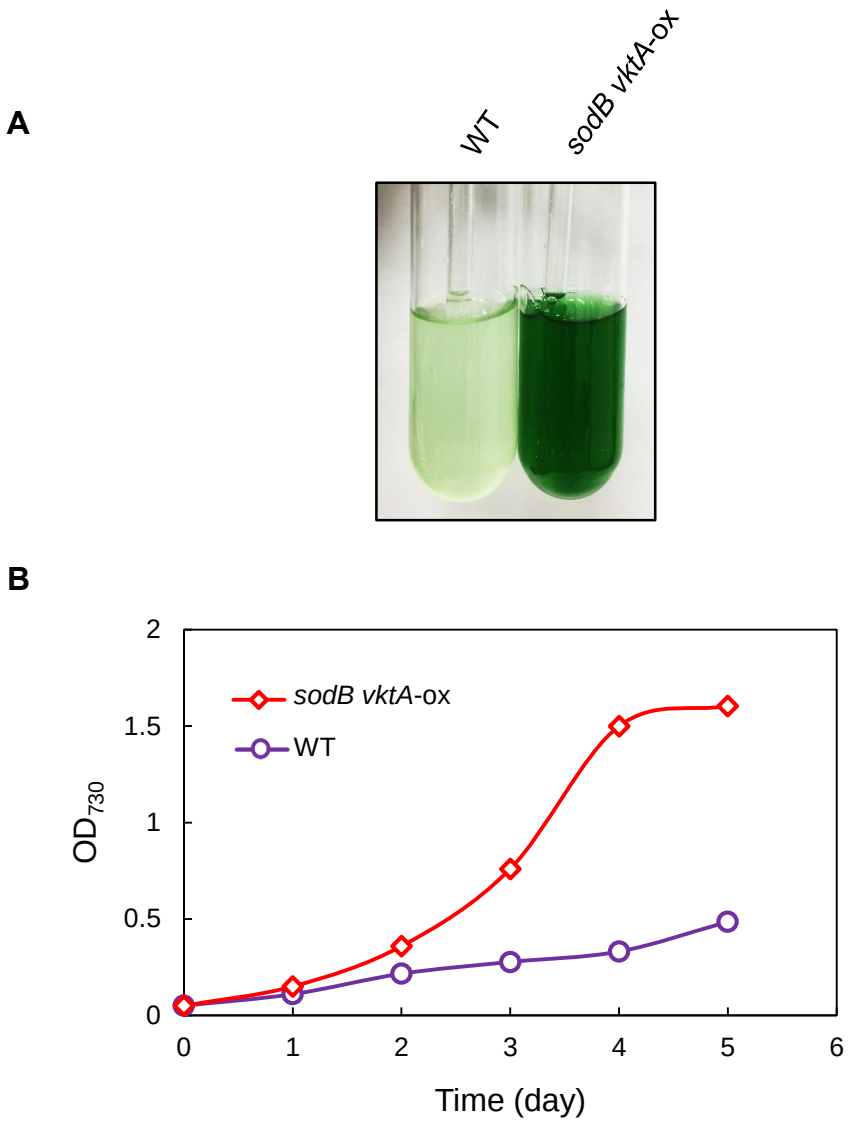

Figure 2.16 Effects of the overexpression of Fe-SOD plus VktA on the on the growth curve in *Synechococcus* under oxidative stress. Wild-type (WT) and transformed cells were grown under normal growth condition in the presence of 1.5 µM methyl viologen. The photos were taken after 5 days (A). Growth of cells was monitored in terms of optical density at 730 nm (B).

2.5 Discussion

2.5.1 Roles of SOD and catalase in the protection of PSII from photoinhibition

The present study showed that overexpression of Fe-SOD and VktA in *Synechococcus* alleviated the photoinhibition of PSII, indicating that SOD and catalase protect PSII from photoinhibition. Overexpression of VktA alone alleviated the photoinhibition of PSII, but the protective effect was limited to approximately 10% (Fig. 2.7), as observed previously by Jimbo *et al* (2013). The minimal effect of VktA on photoinhibition might have been due to the accumulation of O_2^- in VktA-overexpressing cells under strong light, even though intracellular levels of H_2O_2 fell significantly (Fig. 2.9). Overexpression of Fe-SOD alone alleviated the photoinhibition of PSII to a greater extent than overexpression of VktA alone (Fig. 2.7). The more pronounced effect might be explained by the effective decomposition of O_2^- in Fe-SOD-overexpressing cells, even though intracellular levels of harmful H_2O_2 did increase (Fig. 2.9). Overexpression of both Fe-SOD and VktA together alleviated the photoinhibition of PSII much more effectively (Fig.2.7), indicating that decreases in levels of both O_2^- and H_2O_2 efficiently protected PSII from photoinhibition. Although I was unable to compare the individual effects of O_2^- and H_2O_2 quantitatively, it is likely that both ROS are able to increase the extent of photoinhibition of PSII. In addition, H_2O_2 can be converted to OH•, the most toxic ROS, via the Fenton reaction in the presence of reduced metal ions, such as Fe^{2+}, which is generated by the reduction of Fe^{3+} by O_2^- (Keyer and Imlay 1996, McCormick et al. 1998). Thus, suppression of the production of both O_2^- and H_2O_2 should also mitigate oxidative stress by depressing the generation of OH•.

The addition of methyl viologen to cells stimulated the photoinhibition of PSII in all four strains examined (Fig. 2.8). In the presence of this compound, the protective effect of

overexpression of VktA was almost equivalent to that of the overexpression of Fe-SOD. It seems likely that enhanced production of O_2^- in the presence of methyl viologen might have increased the accumulation of H_2O_2 and that the capacity to decompose H_2O_2 might, in turn, have become a rate-limiting step. Therefore, the effect of overexpression of VktA might have been enhanced, as compared to that of the overexpression of Fe-SOD that resulted in more pronounced increases in levels of H_2O_2 (Fig. 2.9). In addition, the strong protective effect of overexpression of both Fe-SOD and VktA together was probably due to synergistic abilities of these enzymes to scavenge both O_2^- and H_2O_2.

2.5.2 Actions of ROS in the photoinhibition of PSII

The overexpression of Fe-SOD and VktA did not affect photodamage to PSII but enhanced the repair of PSII, indicating that SOD and catalase might not protect PSII from photodamage but might, rather, protect the repair of PSII (Fig. 2.7). Moreover, the addition of methyl viologen to cells did not accelerate the rate of photodamage to PSII, as observed previously (Nishiyama et al. 2001). These observations support our hypothesis that ROS act primarily by inhibiting the repair of PSII and not by damaging PSII directly (Nishiyama et al. 2001, Nishiyama et al. 2004, Nishiyama et al. 2011). They are also consistent with the "two-step mechanism" of photodamage to PSII. In the two-step mechanism, photodamage to PSII occurs in two steps: primary damage occurs at the oxygen-evolving complex, most probably at the manganese cluster, via absorption of UV and blue light; and secondary damage occurs at the reaction center via absorption of visible light that has been absorbed by chlorophylls (Hakala et al. 2005, Ohnishi et al. 2005). According to this mechanism, ROS are not the primary cause of photodamage to PSII or, at least, they are not responsible for primary damage to PSII (Nishiyama et al. 2006).

2.5.3 Role of SOD and catalase in the protection of protein synthesis from ROS

Under strong light, overexpression of either Fe-SOD or VktA enhanced the synthesis *de novo* of the D1 protein, which is required for the repair of PSII, while the overexpression of both Fe-SOD and VktA together enhanced the synthesis of D1 to a much greater extent (Fig. 2.11). The synergistic effects of the two enzymes might again be explained by decreased levels of both O_2^- and H_2O_2, as well as by the resultant decrease in levels of OH•. Furthermore, I observed that not only was the synthesis of the D1 protein enhanced but the synthesis of almost all the proteins in thylakoid membranes was also enhanced. This observation suggests that the protein-synthetic machinery might itself have been protected from damage by ROS.

The particular sensitivity of the protein-synthetic machinery to ROS was demonstrated in studies of the effects of elevated levels of ROS on protein synthesis in *Synechocystis* (Nishiyama et al. 2001, Nishiyama et al. 2004). Analysis of polysomes in *Synechocystis* revealed that ROS inhibit the synthesis of the D1 protein *de novo* at the elongation step of translation (Nishiyama et al. 2001, Nishiyama et al. 2004). Moreover, within the translational machinery, elongation factors EF-G and EF-Tu were identified as the targets of ROS in *Synechocystis*. In *Synechocystis*, EF-G is inactivated via the oxidation, by ROS, of two specific cysteine residues, with subsequent formation of an intramolecular disulfide bond (Kojima et al. 2007, Kojima et al. 2009), while EF-Tu is inactivated via oxidation of the single cysteine residue (Cys82) with subsequent formation of an intermolecular disulfide bond and sulfenic acid (Yutthanasirikul et al. 2016). The particular sensitivity of EF-G to ROS has also been demonstrated in *E. coli* (Nagano et al. 2012, Nagano et al. 2015). It remains to be determined whether overexpression of Fe-SOD and VktA can suppress the oxidation of these elongation factors under strong light, thereby protecting protein synthesis from ROS-induced inhibition.

2.5.4 Conclusions and perspectives

Overexpression of both Fe-SOD and VktA mitigates the photoinhibition of PSII by protecting the repair of PSII rather than by protecting PSII from photodamage. Such protection might be achieved by the synergistic effects of efficient scavenging of both O_2^- and H_2O_2 with the consequent protection, from oxidative damage, of the synthesis of the proteins that are required for the repair of PSII. Reinforcement of the capacity to scavenge O_2^- and H_2O_2 with Fe-SOD and VktA might allow improvements in the tolerance of the photosynthetic machinery to strong light, as well as to other abiotic stressors that engender oxidative stress.

Chapter 3

Conclusions and perspectives

Light is a driving force of photosynthesis, but excess light is also harmful to photosynthetic organisms that include plants and cyanobacteria. Absorption of excess energy accelerates the production of ROS, such as $^1O_2$, O_2^-, H_2O_2, and OH•, which cause photo-oxidative damage. Oxidative damage is often associated with photoinhibition of PSII, due to the particularly sensitivity of the repair of PSII to ROS. ROS inhibit the repair of PSII by suppressing the synthesis *de novo* of proteins that are required for the repair of PSII, such as the D1 protein, which constitutes the reaction center of PSII. Recent studies found that ROS suppress the synthesis of proteins at the elongation step of translation and that specific elongation factors, EF-G and EF-Tu, are the primary targets of ROS (Nishiyama et al. 2011, Nagano et al. 2012, Yutthanasirikul et al. 2016).

To improve the defense mechanisms of photosynthesis against photo-oxidative stress, several strategies are available. One of the most effective strategies is to strengthen the capacity of antioxidative systems to scavenge ROS rapidly and effectively. Photosynthetic organisms have developed two types of antioxidative systems, namely, ROS-scavenging enzymes and non-enzymatic antioxidants, in order to cope with oxidative damage. SOD is considered as the first line of the antioxidative systems, which can powerfully scavenge O_2^- to H_2O_2 and O_2, with the consequent protection from oxidative damage. Cyanobacteria as well as other photosynthetic organisms possess several distinct types of SOD, which differ according to its metal cofactor. Fe-SOD and Mn-SOD are major SOD in cyanobacteria. Fe-SOD is localized in the cytosol, while Mn-SOD is localized both in the cytosol and thylakoid membranes.

Overexpression of Fe-SOD from *Synechocystis* or Mn-SOD from *Anabaena* sp. PCC 7120 in cyanobacterium *Synechococcus* elongatus PCC 7942 increased the tolerance of PSII to strong light to a similar extent. However, when stress was strengthened by combination of strong light and methyl viologen-mediated oxidative stress, overexpression of Fe-SOD conferred higher tolerance of PSII than that of Mn-SOD (Fig. 2.4). Overexpression of either

Fe-SOD or Mn-SOD mitigated the photoinhibition of PSII by protecting the repair of PSII rather than by protecting PSII from photodamage. This protection might be achieved by the efficient scavenging of O_2^-. However, it is still unknown why overexpression of Fe-SOD is more effective than that of Mn-SOD under more severe photo-oxidative stress. I selected Fe-SOD for further studies due to its high activity against photo-oxidative stress.

Overexpression of Fe-SOD in cells enhanced the scavenging capacity of O_2^- and, in concurrently, it rapidly induced the production of H_2O_2. Overexpression of Fe-SOD increased intracellular levels of H_2O_2 under strong light, which were even higher than those in wild-type cells under combination of strong light and methyl viologen-mediated stress. However, the decreased levels of O_2^- might be sufficient to mitigate the photoinhibition of PSII by enhancing the repair of PSII but not by diminishing photodamage to PSII, even though the levels of H_2O_2 were raised. This result also demonstrates that O_2^- is a factor that inhibits the repair of PSII with the consequent acceleration of the photoinhibition of PSII.

Decrease in intracellular levels of H_2O_2 was also achieved by introduction of a highly active catalase VktA from *Vibrio rumoiensis* S-1^T. This catalase was isolated from an H_2O_2-rich drain pool of a fish-processing plant. Overexpression of VktA protected the repair of PSII without affecting photodamage to PSII under the combination of strong light and methyl viologen-mediated oxidative stress. The resultant decrease in the levels of H_2O_2 was sufficient to mitigate the photoinhibition of PSII by accelerating the repair of PSII, but the protective effect was as limited as 10%. This limited effect might be explained by the toxicity of the accumulation of O_2^-, which is the first ROS that is rapidly produced from photosynthetic electron transport under strong light.

Overexpression of both SOD and catalase successfully reduced intracellular levels of O_2^- and H_2O_2, which can also prevent the generation of OH•. Due to the instability and short life, there is no appropriate method to measure intracellular levels of OH•. Overexpression of

Fe-SOD and VktA together acts synergistically to decrease overall intracellular levels of ROS and to protect the repair of PSII without affecting photodamage to PSII under strong light. Thus, overexpression of SOD and catalase together is more effective in protection of PSII from photoinhibition than individual overexpression of SOD or catalase.

Analysis of protein synthesis by monitoring the incorporation of ^{35}S-labeled methionine and cysteine into cells under strong light suggested that decrease in levels of ROS mitigated photoinhibition by protecting the repair of PSII via enhancing the synthesis *de novo* of proteins and, in particular, the D1 protein. Overexpression of both Fe-SOD and VktA had synergistic effects on protein synthesis *de novo*. Liquid-scintillation counting also indicated that the double overexpression is much more effective to protect protein synthesis under strong light. Thus, my study provides the knowledge of the effects of the overexpression of SOD and catalase on the repair of PSII and protein synthesis under photo-oxidative stress in *Synechococcus*.

The protection of the repair of PSII might depend on the improved protection of the protein-synthetic machinery from oxidative damage. As mentioned above, specific elongation factors, EF-G and EF-Tu, have been identified as critical targets of ROS in the protein-synthetic machinery. Therefore, it remains to be determined whether the overexpression of SOD and catalase protect EF-G or EF-Tu from oxidation by ROS. Future studies should be directed to monitor the redox state of these elongation factors in order to understand the mechanisms by which the synthesis of the D1 protein synthesis is enhanced by overexpression of Fe-SOD and VktA. Moreover, since organisms manage to cope with photo-oxidative stress by use of various mechanisms, alteration of one process might affect overall systems against photo-oxidative stress. Thus, it remains to be elucidated how the overexpression of Fe-SOD and VktA affects other antioxidative systems, such as antioxidants. In addition, it remains to be clarified whether

this strategy is applicable to other types of environmental stress or other photosynthetic organisms. Thus, future studies should be directed to address the following subjects.

1. Effects of the overexpression of Fe-SOD and VktA on the protection of EF-G or EF-Tu from oxidative damage

Overexpression of SOD and catalase protects the synthesis of the D1 protein from oxidative damage by ROS. Analysis of polysomes in *Synechocystis* demonstrated that ROS inhibit the synthesis of the D1 protein at the elongation step of translation (Nishiyama et al. 2001, Nishiyama et al. 2004). Furthermore, within the translational machinery of *Synechocystis*, elongation factors EF-G and EF-Tu were identified as the targets of ROS. In *Synechocystis*, EF-G is inactivated via the oxidation, by ROS, of two specific cysteine residues, with subsequent formation of an intramolecular disulfide bond (Kojima et al. 2007, Kojima et al. 2009). EF-Tu is inactivated via oxidation of the single cysteine residue (Cys82) with subsequent formation of an intermolecular disulfide bond and sulfenic acid (Yutthanasirikul et al. 2016). The particular sensitivity of EF-G to ROS has also been demonstrated in *E. coli* (Nagano et al. 2012, Nagano et al. 2015). It remains to be determined whether overexpression of Fe-SOD and VktA can su ppress the oxidation of these elongation factors under strong light, thereby protecting protein synthesis from ROS-induced inhibition.

2. Effects of the overexpression of Fe-SOD and VktA on other antioxidative systems

Photosynthetic organisms have developed effective antioxidative systems to scavenge ROS, which include ROS-scavenging enzymes, such as SOD, catalase, glutathione peroxidase, and ascorbate peroxidase; and also non-enzymatic antioxidants, such as carotenoids, flavonoids, tocopherol, and glutathione. Individual ROS-scavenging enzymes and antioxidants have different properties and substrate specificities to ROS. They must be well organized to control

the levels of ROS in cells. For example, overexpression of Mn-SOD in maize induced the expression of other ROS-scavenging enzymes, leading to increased tolerance to low-temperature stress (Kingston-Smith and Foyer 2000). Thus, future studies should be directed to unveil whether overexpression of Fe-SOD and VktA affect other antioxidative systems in *Synechococcus*.

3. Effects of the overexpression of Fe-SOD and VktA on the tolerance to other environmental stresses

Since abiotic stresses, such as drought, salinity, and high temperature, cause oxidative damage, they can influence the photoinhibition of PSII. For example, salt stress stimulates photoinhibition of PSII in *Chlamydomonas reinhardtii* (Neale and Melis 1989), *Spirulina platensis* (Lu and Zhang 1999, Lu and Vonshak 2002), *Synechocystis* (Allakhverdiev et al. 2002), corn (Hichem et al. 2009), and Syrian barley (Kalaji et al. 2011). Oxidative damage by salt stress is associated with the production of ROS. However, organisms protect themselves against salt stress by enhancing their antioxidative systems to scavenge ROS (Jiang and Zhang 2002, Patsikka et al. 2002). In nature, the capacity of the antioxidative systems in cells is limited. Therefore, improving the antioxidant systems in cultivars is able to increase the tolerance to salt stress in cotton (Gossett et al. 1996) and drought stress in barley (Acar et al. 2001).

Under abiotic stresses including salt stress, ROS are rapidly generated. For example, H_2O_2 accumulates under salt stress in mangrove (Parida et al. 2004). Excess ROS stimulate the photoinhibition of PSII by inhibiting the repair of PSII, leading to slow growth of photosynthetic organisms and low production of their products. Thus, improving the antioxidant systems by overexpression of Fe-SOD and VktA might greatly improve the ROS-

scavenging capacity, leading to the maintenance of high photosynthetic activity under environmental stress, such as salinity and drought stresses.

4. Effects of the overexpression of SOD and VktA on photoinhibition of PSII in higher plants.

Recently, many studies demonstrated that overexpression of multiple ROS-scavenging enzymes enhance the tolerance to various environmental stresses. For example, overexpression of Cu/Zn-SOD and ascorbate peroxidase together in tall fescue enhanced tolerance under methyl viologen, H_2O_2 and metal stresses (Lee et al. 2007). However, some studies did not show any synergistic effects of multiple overexpression. For example, overexpression of SOD and ascorbate peroxidase in cotton under the combination of moderate chilling and strong light stresses (Payton et al. 2001). Thus, the synergistic effects of co-overexpression of Fe-SOD and VktA in plants on the tolerance to various environmental stresses need to be verified. Moreover, there is limited knowledge whether the overexpression of SOD and catalase can mitigate the photoinhibition of PSII and, in particular, the repair of PSII in plants. The detailed effects of the overexpression of SOD and catalase on photoinhibition in plants also remain to be elucidated at the molecular level.

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Publications

Publications:

1. Sae-Tang, P., Hihara, Y., Yumoto, I., Orikasa, Y., Okuyama, H. and Nishiyama, Y. Overexpressed superoxide dismutase and catalase act synergistically to protect the repair of PSII during photoinhibition in *Synechococcus elongatus* PCC 7942.
   Plant and Cell Physiology (2016) DOI: 10.1093/pcp/pcw110, in press.

2. Ueno, M., Sae-Tang, P., Kusama, Y., Hihara, Y., Matsuda, M., Hasunuma, T. and Nishiyama, Y.
   Moderate heat stress stimulates repair of photosystem II during photoinhibition in *Synechocystis* sp. PCC 6803.
   Plant and Cell Physiology (2016) DOI: 10.1093/pcp/pcw153, in press.

Presentations:

1. Sae-Tang, P., Hihara, Y., Yumoto, I., Orikasa, Y., Okuyama, H. and Nishiyama, Y. Synergistic effects of iron superoxide dismutase and catalase on the protection of photosynthesis to strong light in *Synechococcus elongatus* PCC 7942.
   The 57th Annual Meeting of the Japanese Society of Plant Physiologists, 18-20 March 2016, Iwate, Japan (Oral presentation).

2. Sae-Tang, P., Hihara, Y., Yumoto, I., Orikasa, Y., Okuyama, H. and Nishiyama, Y.
   Overexpressed superoxide dismutase and catalase enhance the protection of photosynthesis to strong light in *Synechococcus elongatus* PCC 7942.
   The 17th International Congress on Photosynthesis Research, 7-12 August 2016, Maastricht, The Netherlands (Poster presentation).